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Characterization of an Estrogen-degrading Culture, *Novosphingobium tardaugens* ARI-1

Yuechauan Yang

To the Graduate Council:

I am submitting herewith a thesis written by Yuechauan Yang entitled "Characterization of an Estrogen-degrading Culture, *Novosphingobium tardaugens* ARI-1." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Environmental Engineering.

Kung-Hui Chu, Major Professor

We have read this thesis and recommend its acceptance:

Chris D. Cox, Paul D. Frymier

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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Anne Mayhew
Vice Chancellor and
Dean of Graduate Studies

CHARACTERIZATION OF AN ESTROGEN-DEGRADING CULTURE, *NOVOSPHINGOBIUM TARDAUGENS* ARI-1

A Thesis Presented for the

Master of Science

Degree

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Yuechuan Yang

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ABSTRACT

In this study, experiments were conducted to characterize the bacterium *Novosphingobium spp* ARI-1, which is the only known estradiol-degrading strain. The kinetic parameters, the effects of acetone on cell growth, and the effects of starvation on estrogen degradation have been measured. A quantitative fingerprinting molecular technique, called real-time-t-RFLP, was used to characterize microbial communities of activated sludge samples collected from six wastewater treatment plants in east Tennessee. The experimental data showed that ARI-1 was capable of growing on estrogens in the presence and absence of acetone. In the absence of acetone, ARI-1 grew on 17α-estradiol, 17β-estradiol, and estrone with cell yields Y=0.05-0.13 (mg VSS/mg estrogen). The half-velocity constant (K_m) was 7.8±2.3mg/L for 17β-estradiol and 5.1±1.5mg/L for estrone. The maximum specific substrate utilization rate ($\hat{q}$, mg estrogen/mg VSS-day) was 14.0±4.5d^{-1} for 17β-estradiol and 14.0±4.4d^{-1} for estrone. Degradation rates decreased when ARI-1 was deprived of estrogen for 1, 3, and 7 days. After growing in LB (nutrient-rich) medium without exposure to 17β-estradiol for 7 days, ARI-1 degraded 17β-estradiol at a much slower rate and failed to degrade estrone for more than 10 days. The results of microbial community analysis of activated samples suggested but not confirmed the presence of ARI-1. The results of this study, including the kinetic parameters of estrogen degradation by ARI-1, starvation effects, and prevalence of ARI-1 in various activated sludge system, are valuable for engineers to design a biological treatment system for enhanced estrogen removal.

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CHAPTER ONE

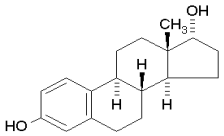
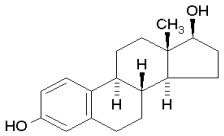
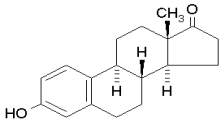
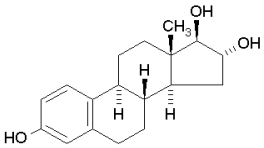
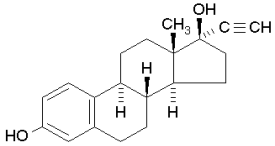
INTRODUCTION

1.1 Introduction

Estrogens, containing steroidal structures, are endogenous hormones. Estrogens play an important role in a variety of fundamental biological processes, particularly in the reproductive system in females (Stumpf 1968; O'Byrne et al., 1993). Estrogens can be produced naturally and synthetically. Natural estrogens include 17β-estradiol, 17α-estradiol, estrone, and estriol; while the synthetic estrogens include 17α-ethynylestradiol, which is commonly used for birth control. Women excrete 17β-estradiol (a potent endogenous estrogen) in female ovaries, ranging from 10 to 100μg/day. The quantity produced depends on age, reproductive status, and days of the menstrual cycle. Pregnant women can excrete as much as 30mg of 17β-estradiol per day (Johnson et al., 1999). Men also excrete 2 to 25μg of 17β-estradiol daily (Williams et al., 1996). Table 1.1 summaries the estrogens, their structures, and their properties.

Estrogenic effects, such as the alteration of sexual differentiation, offspring sex ratio, abortion, breast cancer (Henderson et al., 1993), and testicular cancers (Osterlind 1986; Brown et al., 1986) have been reported. These estrogenic effects have drawn worldwide attention over the past few years. It is generally accepted that estrogens may be responsible for the sexual disruption in some wild life populations (Guillette et al., 1994; Fox 1992), which includes feminization in male fish (Pelissero et al., 1993; Christiansen

***Table 1.1* Properties of estrogens**

Name	Structure Image	Molecular weight (g/mol)	Water solubility (mg/L at 20°C) [1]	Log K_{ow} [1]	Relative estrogenic potencies		
					YES [2]	MVLN [3]	VTG [4]
17α-estradiol ($C_{18}H_{24}O_2$)		272.38	-	4.01	-	-	-
17β-estradiol ($C_{18}H_{24}O_2$)		272.38	13	3.94	1	1	1
Estrone ($C_{18}H_{22}O_2$)		270.37	13	3.43	0.4 [5]	0.2 [5]	0.8 [5]
Estriol ($C_{18}H_{24}O_3$)		288.39	13	2.81	-	0.083 [7]	-
17α-ethynyl estradiol ($C_{20}H_{24}O_2$)		296.40	4.8	4.15	0.9 [5] (0.96) [6]	-	30.6 [5] (0.82) [6]

[1] Jurgens, et al., 1999; Tabak, et al., 1981; Meylan and Howard 1995; Schweinfurth 1996; Norpoth, et al., 1973; Johnson, et al., 1998; Lai et al., 2000.
[2] YES, yeast estrogenic screening assay (YES-screen.)
[3] MVLN, MVLN-assay (transformed MCF-7 human breast cancer cell line).
[4] VTG, plasma vitellogenin.
[5] Van den Belt, et al., 2003.
[6] Segner, et al., 2003.
[7] Gutendorf and Westendorf, 2001.

et al., 2002; Hansen et al., 1998). The presence of estrogens in aquatic environments are implicated in potential human health problems (Panter et al., 2000).

Significant amounts of estrogens can be introduced into the environment through run-off sewage sludge used for agriculture (López de Alda, 2001) and wastewater treatment plant (WWTP) effluent outfalls (Andersen et al., 2003). In the United Kingdom, Netherlands, Germany, Italy, Canada, Brazil, and Japan, estrogens have been found in rivers, in treated water, and even in spring water (Desbrow et al., 1998).

Recently, estrogens have been classified as micropollutants (Janssens et al., 1997), occurring in the environment with concentrations on the order of nanograms per liter (López de Alda et al., 2001). Recent studies have reported the detection of estrogens in the effluent from municipal WWTPs (Desbrow et al., 1998; Routledge et al., 1998; Harries et al., 1997; Folmar et al., 1996) and the estrogen concentrations are in the range of 0.1-1ng/L (Routledge et al., 1999; López de Alda et al., 2001). Estrogen removal efficiency in WWTPs has been estimated from 50% to 90% (Baronti et al., 2000; Nasu et al., 2001).

Biodegradation may be an important removal mechanism for estrogens in WWTPs (Layton et al., 2000). Recently, the only known estrogen-degrading microorganism, *Novosphingobium tardaugens* ARI-1 (ARI-1), has recently been isolated from activated sludge in Japan (Fujii et al., 2002), and its characteristics have not been fully measured. Since the environmentally occurring 17β-estradiol is in the concentration range of nanograms per liter (López de Alda et al., 2001), it is important to determine whether or not ARI-1 is capable of degrading 17β-estradiol at these relevant concentrations.

1.2 Research Goal and Objectives

In this study, experiments were conducted to achieve the following objectives:

1. Determine degradation kinetics of 17α-estradiol, 17β-estradiol and estrone;
2. Examine the effects of nutrient-rich medium starvation on 17β-estradiol degradation;
3. Examine the effects of complex medium on 17β-estradiol degradation;
4. Examine the effects of estrogen mixtures on estrogen degradation;
5. Examine the prevalence of ARI-1 in various activated sludge systems.

CHAPTER TWO

LITERATURE REVIEW

2.1 Known Characteristics of *Novosphingobium tardaugens* ARI-1

ARI-1 was isolated from activated sludge samples that were collected in Japan (Fujii et al., 2002). The sludge samples were enriched with 30mg/30mL of 17β-estradiol on a yeast nitrogen base without any amino acid supplement (E2-YNB medium) at 25^{o}C, 150rpm (Fujii et, al., 2002). After degradation activity of 17β-estradiol was observed, the enriched culture was subcultured three times and streaked on nutrient agar for isolation of a pure estrogen-degrading culture, which was named ARI-1 (Fujii et al., 2002). ARI-1 also was capable of degrading 10mg/mL of 17β-estradiol in E2-YNB medium supplemented with 0.5% (w/v) of yeast extract (or 0.05% glucose). ARI-1 can degrade estrone, estriol, and 17β-estradiol but not 17α-ethynylestradiol. No metabolites during the estrogen degradation were observed. Based on 16S rDNA sequence (DDBJ database under accession no. AB070237), ARI-1 belongs to the *Novosphingobium spp* (Fujii et al., 2002).

2.2 Degradation Kinetics

Monod kinetics

Monod kinetics (Monod 1949) are used in this study. The specific growth rate (μ) is a function of maximum specific growth rate (μ_m), substrate (S) concentration, and half-velocity (K_m) as shown in Equation (2-1):

$$\mu = \frac{\mu_m S}{K_m + S} \tag{2-1}$$

where μ_m is the maximum specific growth rate, time^{-1};

μ is the specific growth rate, time^{-1};

S is the substrate concentration in the solution, mass/unit volume;

K_m is the saturation constant or half velocity constant, mass/unit volume.

K_m is the concentration when the specific rate of growth is equal to one-half of the maximum specific growth rate.

In Equation (2-1), μ_m and K_m are essential parameters since they describe how fast the bacteria can grow and the affinity toward the substrate. In a biological treatment system, bacteria with large μ_m values exhibit fast growth and they are less likely to be subject to washout; small K_m values represent high substrate affinity and bacteria with small K_m values are likely to be the dominant species in the system. The study of the kinetics can provide necessary information about the bacteria and allow us to predict their behavior in a biological treatment system. The maximum specific growth rate can be written as:

$$\mu_m = \hat{q} \cdot Y \tag{2-2}$$

where μ_m is the maximum specific growth rate, time^{-1};

$\hat{q}$ is the maximum substrate utilization rate, time^{-1};

Y is the cell yield, mass of cells produced / mass substrate utilized.

Yields

Yields are defined as the change of cell mass over the change of substrate mass:

$$Y = -\frac{\Delta X}{\Delta S} = \frac{-(X_0 - X_t)}{S_0 - S_t} \tag{2-3}$$

where ΔX is the change of biomass, X_0-X_t;

X_0 is concentration of biomass at time zero;

X_t is the concentration of biomass at time t;

ΔS is defined as the change of BOD_5, S_0-S_t;

S_0 is the substrate concentration at time zero;

S_t is the substrate concentration at time t.

Doubling time

Doubling time (τ_d) is defined as the time necessary for the microorganisms to double biomass:

$$\tau_d = \frac{\text{Ln2}}{\mu} = \frac{0.693}{\mu} \tag{2-4}$$

where τ_d is the doubling time of cell mass;

μ is the specific growth rate (refer to Equation 2-1).

2.3 Molecular Tools

Various molecular tools are currently developed to overcome the disadvantages of traditional methods for the analysis of microorganisms. These molecular techniques directly gather the genetic information of the microorganisms as well as biochemistry of the organism's metabolism (Dionisi et al., 2003; Harms et al., 2003; Bach et al., 2002; Rittmann et al., 2001). In this section, three novel molecular methods, real-time PCR, terminal restriction fragment length polymorphisms (t-RFLP), and real-time-t-RFLP will be described briefly.

2.3.1 Real-time PCR

Real-time PCR, sometime called quantitative PCR, is a fluorescence-based kinetic PCR method. A fluorescent signal, which can be measured at the end of each PCR cycle, increases in proportion to the amount of PCR product (Giulietti et al., 2001; Schnell and Mendoza, 1997; Jung et al., 2000; Bach et al., 2002; Bustin 2000). By comparing the fluorescent signal to standard curves, the initial quantity of gene of interest can be calculated.

Real-time PCR has been applied to detect viruses (Jung et al., 2000), to quantify the *Varicella zoster* virus (Hawrami and Breuer, 1999), to detect pathogens for food quality control (Yang et al., 2003), and to quantify expression of a target gene (Jung et al., 2000; Giulietti et al., 2001). Recently, applications of real-time PCR have been extended to environmental samples, including soil (Hermansson et al., 2001), water (Bach et al., 2002), and activated sludge (Hall et al., 2002; Dionisi et al., 2003; Harms et al., 2003).

2.3.2 Terminal Restriction Fragment Length Polymorphisms (t-RFLP)

Terminal restriction fragment length polymorphisms (t-RFLP) is a finger printing method developed by Liu et al. (1997). In this technique, fluorescent-labeled primers are used during PCR amplification and the amplified PCR product is digested to generate a profile of fragment lengths. The profile represents the relative abundance of microbial species in a microbial community (Liu et al., 1997). The advantage of this technique is its speed and high sample output (Dunbar et al., 2000), which allow for the quick analysis of replicated experimental samples. The drawback of this technique is that different species might contribute to the sample fragment length (Liu et al., 1997; Osborn et al., 2000).

2.3.3 Real-time-t-RFLP

Real-time-t-RFLP was recently developed by the combination of real-time PCR and t-RFLP. This new technique allows for a quick analysis of environmental microbial community structures (Liu et al., 1997; Dunbar et al., 2001; Blackwood et al., 2003; Moeseneder et al., 1999; Yu et al., 2003).

2.4 Activated Sludge Systems

In 1914, Fowler, Andern, and Lockett described some basic features of the activated sludge process (Andern and Lockett 1914). Since then, many activated sludge plants have been constructed in large cities throughout the world. The activated sludge process is an aerobic process for biological wastewater treatment. The basic concept of biological treatment can be described by a stoichiometric equation that relates substrate utilization, cell synthesis, and endogenous respiration (Weston and Eckenfelder 1955). However, the principles, developed based on pure cultures growing on pure substrates, are not directly pertinent to the activated sludge process (Ganczarczyk 1983).

The goals of biological treatment are oxidation of biodegradable organic substances and biomass settling from the treated effluent. Some studies showed that bacteria, fungi, protozoa, and rotifers are present in the activated sludge flocs. (Weddle and Jenkins, 1971; Hanel 1988; Tomlinson and Williams 1975; Curds 1982; Doohan et al., 1975). Most of these microorganisms are held together within flocs in the activated sludge system (Ganczarczyk 1983). The major components of the activated sludge flocs are bacteria and they are responsible for the oxidation of organic substances. The majority of

the bacteria present in the activated sludge are heterotrophic bacteria, which are characterized in Table 2.1.

For activated sludge systems, bacterial communities are always composed of multiple species; some species will quickly replace decimated species. This characteristic is the primary reason for the operational stability of activated sludge systems. However, due to this unique feature of an activated sludge system, subtle changes in the treatment parameters, such as the hydraulic retention time (HRT), the solid retention time (SRT), the pH, the temperature, and the nutrient or dissolved oxygen (DO) level, can result in a major change of the microbial community structure.

Besides the biochemical oxygen demand (BOD) loading, other factors such as DO, nitrogen, and micronutrients are required in the activated sludge system. The oxygen

Table 2.1 **Typical values for heterotrophic bacteria in activated sludge system**

Symbol	Parameter	Unit	Typical Values[1]
Y	Yield	mg VSS[2]/ mg BOD_5	0.45
$\hat{q}$	Maximum substrate utilization rate	mg BOD_5/mg VSS-d	20
μ_m	$\mu_m = Y\hat{q}$, maximum specific growth rate	mg VSS[2]/mg BOD_5·day	9
K_m	Half-saturation constant	mg BOD_5/L	1 (simple substrate); >10 (complex substrate)
b	Decay rate	day^{-1}	0.15
θ_x^{min}	Minimum solids retention time	day	0.11

[1] Typical values adapted from Rittmann and McCarty (2001).
[2] VSS: volatile suspended solids.

consumption rate, the nutrient consumption rate, and the consumption rate of BOD should be proportional to the rate of donor-substrate utilization and endogenous biomass decay (Rittmann and McCarty 2001). Since aerobic and anaerobic bacteria may be present in the treatment system at the same time, changes in DO level can greatly affect the microbial community structure. Additionally, nitrogen and phosphorus as well as some mineral nutrients are very important for the biological treatment since they are all directly or indirectly involved in the biosynthesis. The change of these nutrients may result in treatment problems, including the occurrence of filamentous organisms, which may cause bulking and foaming (Wood and Tchobanoglous 1975).

The SRT is an important parameter for controlling the efficiency of wastewater treatment and its physical and biological characteristics. A longer retention time tends to give better substrate removal efficiency. However, other factors, such as the consumption of soluble microbial products and the sludge settling characteristics, are usually more important.

There are five basic process configurations, and their properties are summarized in Table 2.2.

Table 2.2 **Summary of activated sludge configurations**

Physical Configuration	Properties[1]
Plug Flow (Conventional)	High oxygen demand near the inlet end Possible anoxic conditions with depletion of DO Inhibitors slow down the treatment processes
Step Aeration	High mixed liquor suspended solids (MLSS) Dilution of influent contaminants Spreading out the oxygen-uptake rate along the reactor
Completely Mixed	Spreading of the wastewater uniformly throughout the treatment system Simple system to analyze Favorable for wastewaters containing biodegradable materials Modest concentration toxins to microorganisms
Contact Stabilization	Reduction of overall reactor volume Requires substantially more operational skill and attention Effluent quality is susceptible to sudden changes of loading in small contact tank
Activated Sludge with a Selector	Selection of ecology of the activated sludge by discouraging the overgrowth of undesirable filamentous microorganisms Overcomes sludge bulking problems

[1] Adapted from Rittmann and McCarty, 2001.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals

17α-estradiol (1,3,5[10]-estratriene-3,17α-diol CAS:57-91-0), 17β-estradiol (1,3,5[10]-estratriene-3,17β-diol CAS:50-28-2), and estrone (CAS:53-16-7) were purchased from Sigma-Aldrich, St. Louis, MO. Acetone was purchased from Fisher Science, Fair Lawn, NJ. DMF (dimethylformamide), and BSTFA (N,O-bis (trimethylsily) trifluoroacetamide) were obtained from Pierce, Rockford, IL. The stock solutions of 17α-estradiol, 17β-estradiol, and estrone (1000mg/L) were prepared in acetone for experimental use. The acetone to 17β-estradiol mole ratio was 3700:1; the carbon-based ratio of acetone-carbon to 17β-estradiol-carbon is 617:1.

3.1.2 ARI-1 Culture and Growth Conditions

The ARI-1 culture, *Novosphingobium tardaugens*, was a gift from Dr. Fujii (Fujii et al., 2002). ARI-1 culture was grown and maintained weekly on nitrate minimal salts (NMS) medium (Chu and Alvarez-Cohen 1996) with 4mg/L of 17α-estradiol, 17β-estradiol, or estrone in the presence of acetone. NMS was prepared as follows: 11.76mM $NaNO_3$, 6.10mM Na_2HPO_4, 0.98mM K_2SO_4, 0.15mM $MgSO_4 \cdot 7H_2O$, 0.05mM $CaSO_4 \cdot 7H_2O$, 0.08mM $FeSO4 \cdot 7H_2O$, 1.2μM KI, 2.16μM $ZnSO_4 \cdot 7H_2O$, 1.98μM $MnSO_4$, 1.61μM H_3BO_3, and 7.09μM $CoSO_4$. The pH of NMS was adjusted to 7.5 by adding 10mM of H_2SO_4.

Weekly culture maintenance was conducted by transferring 10μL of cells suspension into new glass vials. The ARI-1 culture was grown in a 40mL glass vial containing 10mL NMS medium and 4mg/L of 17α-estradiol, 17β-estradiol, or estrone. The culture was incubated in a 30^{o}C shaker (Environ-Shaker 3597, Melrose Park, ILL) at 150rpm. The long-term storage of ARI-1 cultures was performed by mixing 0.5mL of cell suspension with 0.5mL of 80% glycerol solution (v/v in autoclaved HPLC water) at -80^{o}C.

3.2 Experimental Methods

3.2.1 Growth and Yield

Experiments were conducted to determine the effect of types of estrogens on ARI-1 growth in the presence of acetone. One milliliter of ARI-1 cell suspension was inoculated into 250mL glass bottles containing 100mL NMS medium and 3mg/L of 17α-estradiol, 17β-estradiol, or estrone. In our laboratory at room temperature, the solubilities of 17α-estradiol, 17β-estradiol, and estrone in water were measured at 1.7mg/L, 3.8mg/L, and 1.4mg/L respectively. Biomass (expressed as optical density OD_{600}) was measured every 24 hours. OD_{600} was measured using a spectrophotometer at 600nm (Beckman, DU640B) and correlated with volatile suspended solids (VSS, mg/L) as described in Section 3.3.2 Biomass Measurement.

Experiments were also conducted without acetone. Acetone was removed from the growth medium by heating the solution to 75^{o}C (the boiling point of acetone is 59^{o}C) while purging with nitrogen at 2mL/sec for 15 minutes. The medium was cooled to room temperature before use.

The growth yields (Y, mg VSS/mg estrogen) of ARI-1 on estrogens were estimated from the growth curves by the Equation (2-3) (refer to Section 2.2).

Biomass is expressed as VSS, which was converted from OD_{600} to VSS according to Equation (3-4) in Section 3.3.2. The concentration of substrate was converted into BOD_5. BOD_5 was calculated under the assumption that BOD_5 is equal to 70% of chemical oxygen demand (COD) (refer to Appendix A for calculation of COD).

The doubling times (τ_d, $time^{-1}$) of ARI-1 grown on estrogens were calculated from the exponential growth phase in growth curves.

3.2.2 Kinetics of Estrogen Degradation

ARI-1 cells were initially grown with an estrogen concentration of 3mg/L in the presence of acetone to allow for rapid growth. Cells were harvested during the exponential growth phase for experimental use. A cell suspension was prepared by centrifugation and washing twice with NMS medium before re-suspending in NMS to an OD_{600} of 1.0. Kinetic tests were conducted in a series of EPA glass vials (40mL) containing 10mL of cell suspension and each of estrogens at a concentration of 0.5mg/L, 1mg/L, 5mg/L, or 10mg/L. Zero hour readings were taken right after the spiking of estrogens. Spiked vials were incubated at 150rpm at 30^{o}C for 5 hours before they were sacrificed for the measurement of estrogen concentrations.

A Lineweaver-Burk reciprocal plot, a linearized form of Monod kinetic model, was used to determine the kinetic parameters K_m and μ_m (Lehninger 1975). The Lineweaver-Burk reciprocal plot is defined as follows:

$$\frac{1}{\mu} = \frac{K_m}{\mu_m}\frac{1}{S} + \frac{1}{\mu_m} \qquad (3\text{-}1)$$

In the equation used for linear regression μ and μ_m were replaced with $\mu = q \cdot Y$ and $\mu_m = \hat{q} \cdot Y$ (refer to Equation 2-2 in Section 2.2) in Equation 3-1:

$$\frac{1}{q} = \frac{K_m}{\hat{q}} \frac{1}{S} + \frac{1}{\hat{q}} \tag{3-2}$$

By plotting $\frac{1}{q}$ versus $\frac{1}{S}$, the slope equals to $\frac{K_m}{\hat{q}}$ and y-intercept equals to $\frac{1}{\hat{q}}$. The Lineweaver-Burk plot provides a good estimate for μ_m (or $\hat{q}$ equivalently). Low substrate values contribute to large error since the errors are not symmetric with regard to the data points. Similar linear plots, the Eadie-Hofstee and Hanes-Woolf plots, were also used to calculate the kinetic parameters.

In the equation used for nonlinear regression μ and μ_m were also replaced with $\mu = q \cdot Y$ and $\mu_m = \hat{q} \cdot Y$ (refer to Equation 2-2 in Section 2.2) in Equation 2-1:

$$q = \frac{\hat{q} \cdot S}{K_m + S} \tag{3-3}$$

Sigmaplot (from SPSS, Version 8.0.2) was used in calculating K_m and $\hat{q}$.

3.2.3 Effects of Starvation on 17β-estradiol Degradation

Experiments were also conducted to determine the response of ARI-1 to periods of 17β-estradiol absence. ARI-1 cells were grown as described above in kinetic studies (refer to Section 3.2.2 for details). Washed ARI-1 cells were diluted in a NMS medium to an OD_{600} between 0.3-0.4. The 100mL diluted cell suspensions in 250mL glass bottles were maintained in a 30^{o}C shaker at 150rpm. After one, three, and seven days of incubation in the absence of 17β-estradiol, the 17β-estradiol-starved cells were examined for their ability to degrade 17β-estradiol. The starved cells were transferred into 250mL

glass bottles containing 100mL NMS and 3mg/L 17β-estradiol only. Five-milliliter liquid samples were taken of the liquid every 24 hours for 17β-estradiol measurement.

Additional experiments were designed to determine the degradation ability of ARI-1 after being grown on nutrient-rich medium without 17β-estradiol. ARI-1 was transferred to LB nutrient-rich medium daily for 7 days before harvesting for experimental use. Similar to cell suspension preparation as described earlier, cells were centrifuged and washed with NMS twice before being re-suspended into 100mL NMS containing 3mg/L of 17β-estradiol only. The final OD_{600} was in the range 0.3-0.4. The 17β-estradiol concentrations were monitored every 24 hours.

3.2.4 Activated Sludge from WWTPs in East Tennessee

The Real-time-t-RFLP Assay (Yu et al., 2003) was used to determine if ARI-1 was present in the activated sludge systems operating under several different treatment process designs. Three different activated sludge systems, including a continuous-stirred tank reactor (CSTR) aeration tank, a plug-flow aeration tank, and an oxidation ditch were surveyed for the presence of ARI-1.

Activated sludge samples were obtained from six different WWTPs in the metropolitan area of Knoxville, Tennessee, during this study. The surveys were conducted in September in 2003, during which the average temperature was 25^{o}C. Domestic wastewater is the major wastewater source for all surveyed WWTPs. WWTP1 is located in Powell in East Tennessee. Oxidation ditches are used in WWTP1. WWTP2 is located 10 miles south of downtown Knoxville. Plug-flow aeration tanks, with air supplied all along the reactors are used in WWTP2. WWTP3, WWTP4 and WWTP6, consisting of CSTR aeration tanks, are located in Knoxville. WWTP5 is located 10 miles

west of downtown Knoxville, which is designed to treat 40MGD, currently treats an average flow of 10MGD. The treated water of these WWTPs is discharged directly into the Tennessee River. Figure 3.1 shows the activated sludge sampling locations in each of the WWTPs. Table 3.1 summarizes the operating conditions of each WWTP.

3.3 Analytical Methods

3.3.1 Estrogens Analysis

Estrogen concentrations were determined as described by Layton et al. (2000). Estrogen in liquid samples was extracted with ether. Extracted estrogen in ether was placed under a gentle stream of nitrogen (0.5mL/sec) until all the ether was evaporated. The precipitated estrogens were dissolved in 450μL of DMF and then derivitized by adding 50μL of BSTFA.

Derivitized samples (0.5mL) were injected on an HP 6890 Series gas chromatograph, equipped with a HP 5973 mass selective detector (GC/MS) and an auto injector. Helium at a flow rate of 1mL/min was used as the carrier gas. The oven temperature was initiated at 240^{o}C for 2 minutes with an increase of 10^{o}C/min to 300^{o}C. Standard curves for each of the estrogens, ranging from 10μg/L to 500μg/L, were used for calculating estrogen concentrations in samples. The GC/MS response in the experiments was 10μg/L for 17α-estradiol, 17β-estradiol, and estrone.

3.3.2 Biomass Measurement

The correlation of cell densities (OD_{600}) and the dry cell mass as VSS was conducted as described by Chu and Alvarez-Cohen, 1996. VSS was determined from the difference of weights after drying at 105^{o}C overnight and after combustion at 550^{o}C for 2 hours.

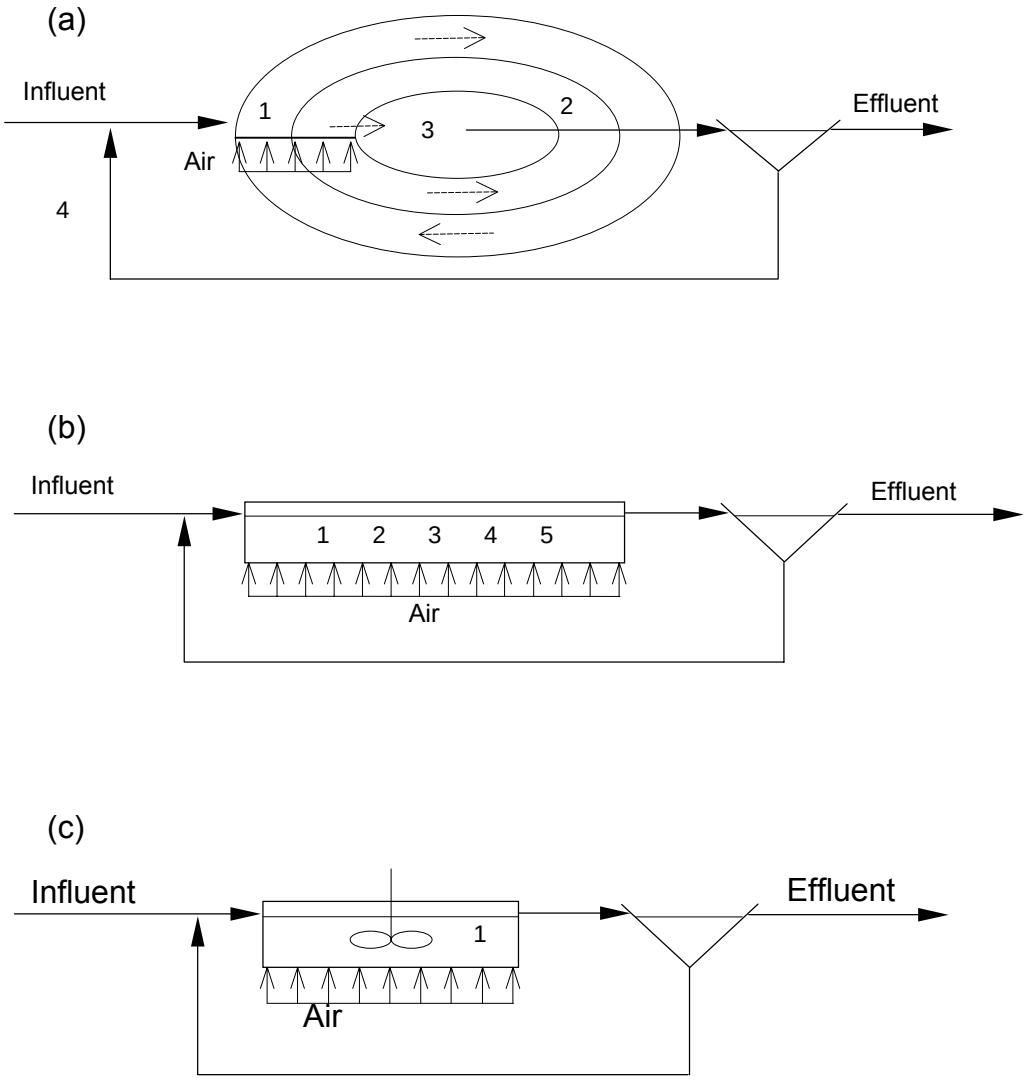


Figure 3.1 **Sampling sites of WWTPs. (a) the sampling sites of the oxidization ditch in WWTP1. (b) the sampling sites of the plug-flow activated sludge system in WWTP2. (c) the sampling site of the CSTR activated sludge systems in WWTP3, WWTP4, WWTP5, and WWTP6.**

Table 3.1 **Comparison of operation conditions of surveyed WWTPs** [1]

	Biological [2] Processes configuration	BOD(mg/L)		DO(mg/L)		SRT (days)	HRT (hours)
		Influent	Effluent	Influent	Effluent		
WWTP1	O	100-300	5-10	1-2	-	10-15	8-10
WWTP2	P	200-300	-	-	-	15-20	10-15
WWTP3	C	200-250	5-10	1-3	-	10-15	15-20
WWTP4	C	200-250	5-10	1-3	-	10-15	15-20
WWTP5	C	100-300	5-10	1-3	7-9	15-20	10-15
WWTP6	C	200-250	-	-	-	15-20	-

[1] ranges were obtained by the monthly reports of each WWTP.
[2] O=oxidation ditch; P=plug-flow reactor; C=continuous-stirred tank reactor.

Conversion of OD_{600} to VSS of the samples were calculated using the following equation (determined from the experimental data, refer to Appendix B for details):

$$VSS(mg/L) = 1726.5 \times OD_{600} + 28.445 \text{, } R^2=0.9831 \quad (3\text{-}4)$$

3.3.3 Real-time-t-RFLP Assay

Real-time-t-RFLP assay was conducted as described by Yu et al., 2003. The total DNA of the activated sludge samples was extracted using the BIO101 Fast DNA Soil Kit (Carlsbad, CA), according to the manufacture's recommendations with a minor modification. Two milliliters of the activated sludge was centrifuged and re-suspended with sodium phosphate buffer (978μL) before being transferred into the lysing tube. 122μL of the MT buffer was added in the lysing tube followed by 30 seconds of beating at a speed of 5.5 scale in the FastPrep Instrument. The lysing tube was then centrifuged at 14,000g for 15 minutes at 4°C, and the supernatant was transferred into a clean Eppis tube before adding 250μL of PPS reagent. Again, the tube was centrifuged and the

supernatant was transferred into a clean 2.0mL Eppis tube before adding 1mL of binding matrix. The Eppis tubes were shaken gently for 10 minutes followed by centrifugation at 12,000g for 5 minutes. The binding matrix was washed with 80% ethanol twice and finally the DNA was re-dissolved in 200μL HPLC water.

The first step of real-time-t-RFLP analysis was real-time PCR reaction, which was conducted with 12.925μL HPLC water, 2.5μL buffer, 0.5μL BSA, 1.5μL $MgCl_2$, 0.2μL dNTP, 0.75μL 1055f (fluorescence labeled), 0.75μL 1392r, 0.625μL probe, 0.25μL Taq, and 5μL of DNA. Real-time PCR standards were prepared using plasmid pCR 2.1 vector (470bp of 16s rDNA, 511bp of intergenic region, and 115bp of 23s rDNA) with a concentration range of 2.29×10^3 to 2.29×10^8 copies in triplicates.

Inhibition of activated sludge in the PCR reaction was avoided by using 10ng of DNA template per PCR reaction based on screening tests. The screening tests were conducted using a series of DNA dilution samples in the PCR reactions. With a DNA loading of 10ng in the PCR reaction, the inhibition of activated sludge was avoided and sufficient DNA was available for the proceeding procedures.

PCR products (352bp) were purified using 1.5% agarose gel electrophoresis. 1.5% gel was prepared by adding 2.25g of agarose in the 150mL TAE buffer (1X). The DNA ladder was prepared with 5μL dye, 15μL H_2O, and 10μL standard, while the samples were prepared with 5μL of the dye and 25μL of the DNA. The expected DNA fragment band in the gel was cut under the UV lamp and was subsequently purified using MicroSpin columns (Amersham Biosciences, Piscataway, NJ).

Recovered PCR products were digested with the restriction enzyme *Msp1*. 40μL DNA was added with 0.5μL *Msp1* enzyme, 5μL 10X buffer, and 5μL 10X BSA. DNA

samples were then centrifuged at 12,000g for 1 minute followed by an overnight incubation in a 37^{o}C water bath. The digested DNA sample (500μL) was precipitated with 3M sodium acetate (50μL) and ethanol (1100μL, 4^{o}C) in an ice bath for 4-5 hours before centrifugation at 12,000g for 15 minutes at 4^{o}C. The DNA pellet was washed with 80% ethanol. The pellet was dried at room temperature for 30 minutes and re-suspended in 50μL HPLC water.

For each t-RFLP reaction, 10μL of formanide, 0.25μL of Rox standard, and 5μL of DNA were added into the t-RFLP reaction tube with the final DNA concentration of 0.2ng/μL. Then the tubes were kept at 95^{o}C for 10 minutes followed by 2 minutes in an ice bath. The terminal fragment lengths of desalted PCR products were analyzed using a model 373A automated sequencer (Applied Biosystems Instruments (ABI), Foster City, CA).

3.3.4 Data Analysis of Real-time-t-RFLP Assay

DNA copies corresponding to a specific fragment (16S rDNA copies/mL) were calculated by the following equation:

$$\frac{\text{16S DNA Copies}}{\text{mL}} = \frac{\text{Area under each peak}}{\sum \text{Area under each peak}} \times \frac{\text{Total DNA Copy Numbers}}{\text{mL}} \qquad (3\text{-}5)$$

In the t-RFLP profile, the peaks with fragment sizes smaller than 30bp were discarded because they were independent of input DNA samples and observed in all samples.

CHAPTER FOUR

RESULTS

4.1 Effect of Acetone on ARI-1 Growth Using Three Different Estrogens

Because ARI-1 was originally isolated using 17β-estradiol in the presence of acetone (Fujii et al., 2002) and because acetone can be used as a carbon source, experiments were conducted to determine the effects of acetone on ARI-1 growth. Figure 4.1 shows the growth curves of ARI-1 on 3mg/L of 17α-estradiol, 17β-estradiol, or estrone in the presence of acetone. ARI-1 grew faster on 17α-estradiol with the maximum biomass of 0.35 expressed as OD_{600}. Estrone resulted in the least growth with a peak OD_{600} of 0.25.

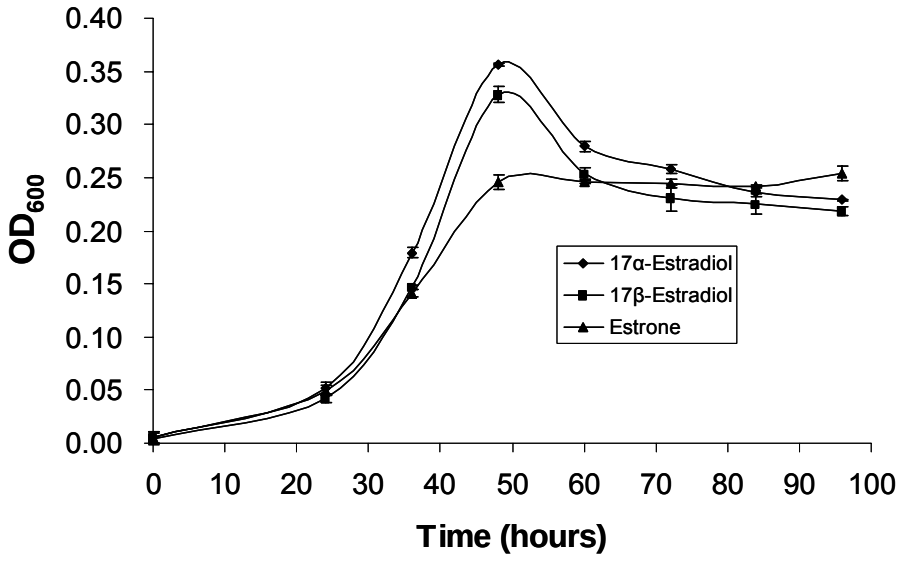


Figure 4.1 **Growth curves of ARI-1 grown on three estrogens in the presence of acetone. The error bars represent the range of duplicates.**

Figure 4.2 shows the growth curves of ARI-1 in the absence of acetone. ARI-1 had the best growth on 17α-estradiol with the maximum biomass of 0.19 expressed as OD_{600}. Estrone gave the lowest growth with a maximum OD_{600} of 0.07. By comparing the growth curves in Figure 4.1 and 4.2, the higher maximum biomass observed in Figure 4.1 was due to the growth on acetone. Table 4.1 summarizes yields (Y, mg VSS/mg estrogen) and the doubling times (τ_d, day) for ARI-1 on three estrogens.

4.2 Kinetics of Estrogen Degradation

Kinetic parameters of ARI-1 on 17β-estradiol degradation were calculated both by linearizing the experimental data by Equation (3-2) and by fitting the non-linear

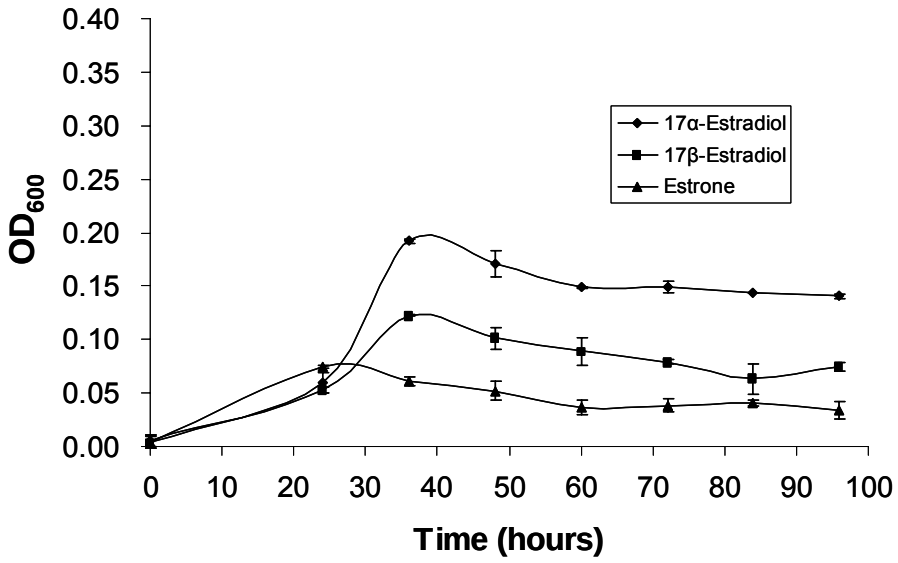


Figure 4.2 **Growth curves of ARI-1 grown on three estrogens in the absence of acetone. The error bars represent the range of duplicates.**

Table 4.1 **Summary of growth parameters of ARI-1**

	17α-estradiol	17β-estradiol	Estrone	Values in Activated Sludge Processed [1]	
				Range	Typical
Y (mgVSS/ mg estrogen)	0.13±0.02[3]	0.08±0.01[3]	0.05±0.01[3]	-	-
Y (mgVSS/ mgBOD$_5$)[2]	0.07±0.01[3]	0.04±0.01[3]	0.03±0.01[3]	0.4-0.8	0.45
τ_d (day)	0.29±0.03[3]	0.35±0.02[3]	0.50±0.02[3]	-	-

[1] data adapted from Wastewater Engineering: Treatment and Reuse, 4th edition, 2003, by Metcalf and Eddy, McGraw Hill.

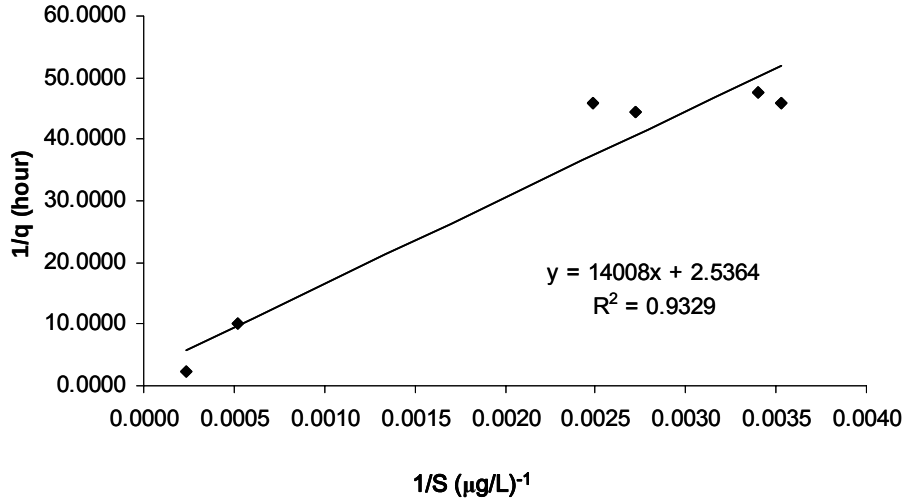
[2] BOD$_5$ was estimated by 70% of COD.
1mg 17α-estradiol/L= 2.7mg/L COD =1.89mg/L BOD$_5$.
1mg 17β-estradiol/L= 2.7mg/L COD =1.89mg/L BOD$_5$.
1mg estrone/L= 2.7mg/L COD =1.89mg/L BOD$_5$ (refer to Appendix A for details).

[3] ± indicate the range of three repeated experiments.

Equation (3-3). Figure 4.3 shows the regression analysis for determining $\hat{q}$ and K_m using both linear and nonlinear regression. As shown in Equation (3-2) the y-axis intersection in Figure 4.3 (a) is equal to the inverse maximum substrate utilization rate, and the slope equals to the K_m over $\hat{q}$. Comparing linear and nonlinear regression, both methods give similar values for K_m and $\hat{q}$.

A similar approach was used to determine the kinetic parameters of ARI-1 for estrone (Figure 4.4). The values of $\hat{q}$ and K_m for estrone determined using Equation (3-2) were similar to the values estimated by Eadie-Hofstee and Hanes-Woolf plots (refer to Appendix B for details). These parameters are summarized in Table 4.2.

(a)



(b)

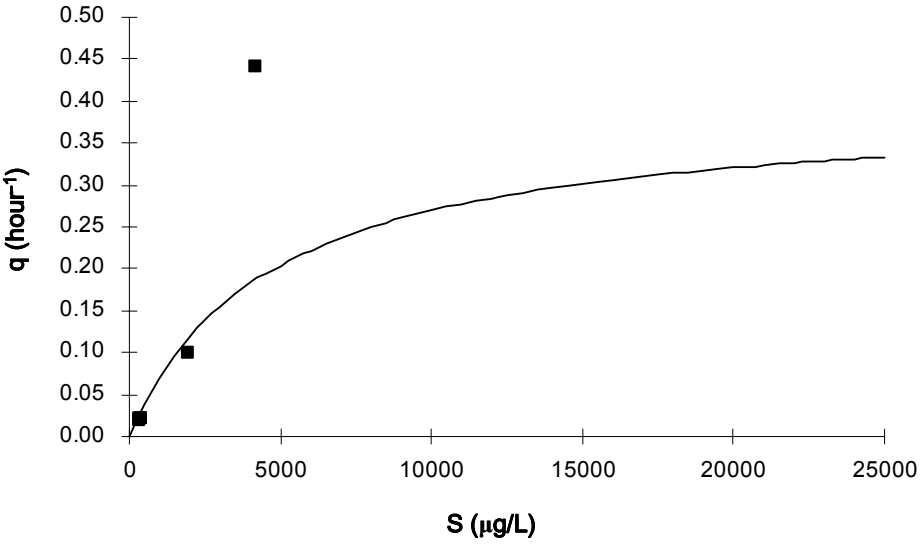


Figure 4.3 **Estimation of kinetic parameters of 17β-estradiol degradation by ARI-1. (a) linear regression, (b) nonlinear regression.**

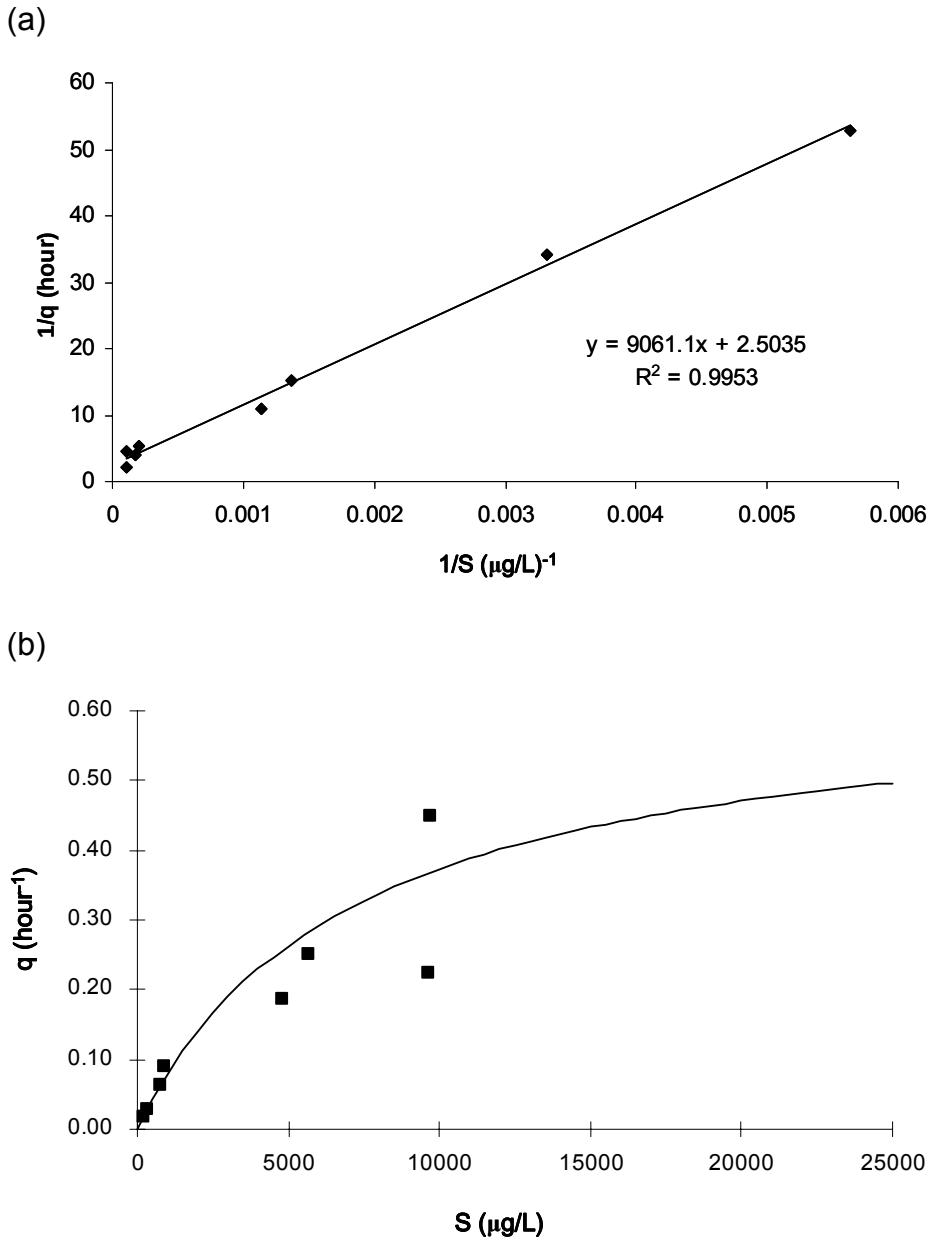


Figure 4.4 **Estimation of kinetic parameters of estrone degradation by ARI-1. (a) linear regression, (b) nonlinear regression.**

Table 4.2 **Summary of kinetic parameters of ARI-1**

	17β-estradiol		Estrone		Valued in Activated Sludge Processes[1]	
	Linear regression	Nonlinear regression	Linear regression	Nonlinear regression	Range	Typical
K_m (mg/L)	7.8±2.3 [3]	5.8±1.1 [3]	5.1±1.5 [3]	8.0±0.9 [3]	-	-
K_m (mg BOD_5/L)[2]	14.7±4.3 [3]	11.0±2.0 [3]	9.6±2.8 [3]	15.1±1.7 [3]	25-100	60
$\hat{q}$ (mg / mgVSS-day)	14.0±4.5 [3]	13.4±2.4 [3]	14.0±4.4 [3]	18.9±3.6 [3]	-	-
$\hat{q}$ (mgBOD_5/ mg VSS-day)[2]	26.5±8.5 [3]	25.3±4.5 [3]	26.5±8.3 [3]	35.7±6.8 [3]	2-10	5
μ_m (mg VSS/ mg estrogen-day)	1.1±0.4 [3]	1.1±0.2 [3]	0.7±0.2 [3]	0.9±0.2 [3]	-	-
μ_m (mg VSS/ mg BOD_5-day)[2]	1.9±0.6 [3]	1.8±0.3 [3]	1.9±0.6 [3]	2.5±0.5 [3]	-	9

[1] data adapted from Wastewater Engineering: Treatment and Reuse, 4th edition, 2003, by Metcalf and Eddy, McGraw Hill.

[2] BOD_5 was estimated by 70% of COD.

1mg 17β-estradiol/L= 2.7mg/L COD =1.89mg/L BOD_5.

1mg estrone/L= 2.7mg/L COD =1.89mg/L BOD_5.

[3] ± indicate the range of three repeated experiments.

4.3 Effects of Starvation on Estrogen Degradation

Since estrogen concentrations in wastewater are typically very low (in the ng/L range), experiments were designed to study the effects of starvation on the estrogens degradation ability of ARI-1. Further, since wastewater contains a wide range of organics and ARI-1 is capable of growing on different carbon sources, experiments were designed to determine whether ARI-1 would retain estrogen degradation ability after being grown on substrates other than estrogens. Experiments were conducted under two different conditions: in the presence of a nutrient-rich medium and in the absence of any carbon source.

4.3.1 Effects of Nutrient-Rich Estrogen-Free Medium

Experiments were conducted to determine the estrogen degradation ability of ARI-1 after being grown on nutrient-rich medium as described in Section 3.2.3. Figure 4.5 shows the biotransformation of 17β-estradiol by ARI-1 cells after being grown on nutrient-rich estrogen-free medium for 7 days (referred as carbon rich-estrogen starved cells later). The 17β-estradiol was depleted quickly in the first 48 hours with a noticeable increase in the estrone concentration. Estrone accumulated in the medium after 48 hours, indicating that ARI-1 lost the ability to degrade estrone after being grown in nutrient-rich medium in the absence of 17β-estradiol. When there was no estrogen starvation period (referred as non-starved cells later), ARI-1 was capable of degrading 17β-estradiol in 9 hours with little or no formation of estrone (Figure 4.6). After 9 hours, both 17β-estradiol and estrone were below the detection limit.

Figure 4.7 shows the specific growth rates of 17β-estradiol by carbon rich-estrogen starved cells and non-starved ARI-1 cells. The specific growth rate for carbon rich-

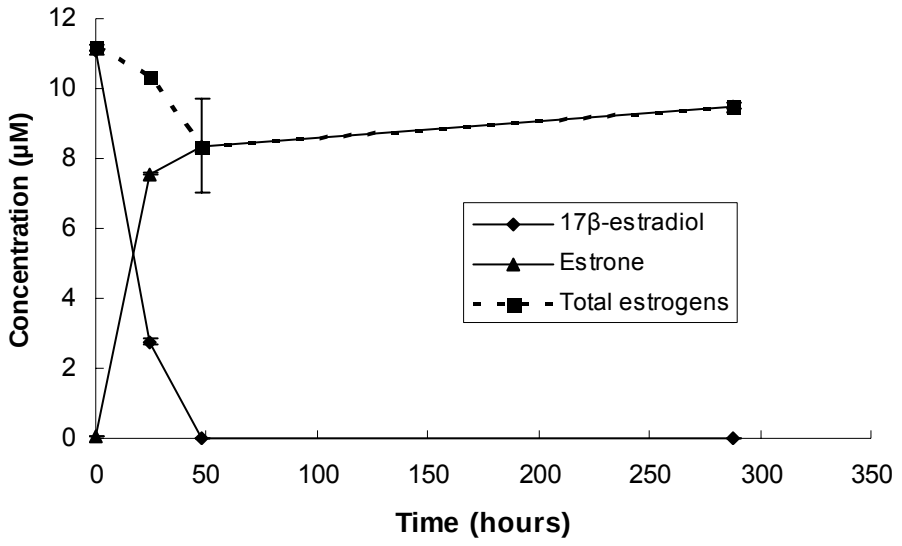


Figure 4.5 **Degradation of 17β-estradiol by ARI-1 grown with nutrient-rich medium without exposure to 17β-estradiol for 7 days. The dashed line shows the total estrogen. The error bars represent the range of duplicates.**

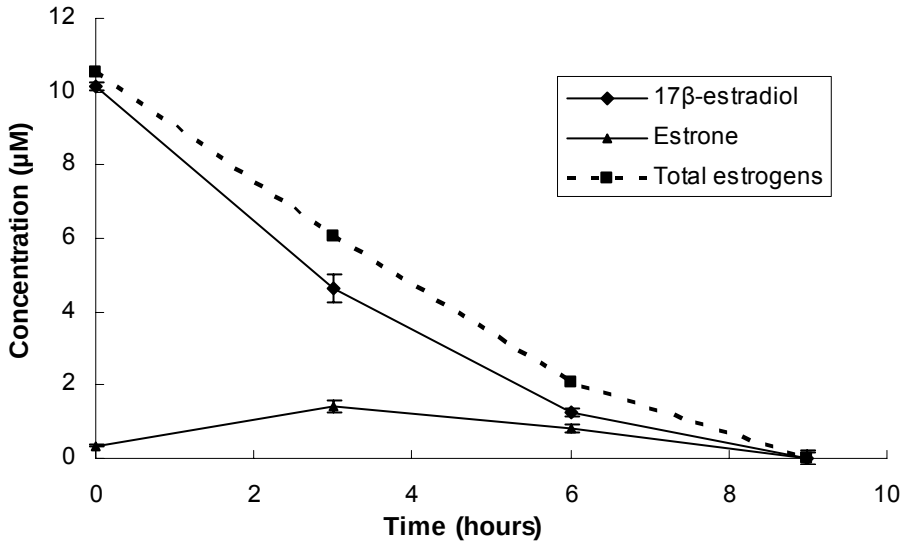


Figure 4.6 **Degradation of 17β-estradiol by ARI-1 without being starved for 17β-estradiol (non-starved cells). The error bars represent the range of duplicates.**

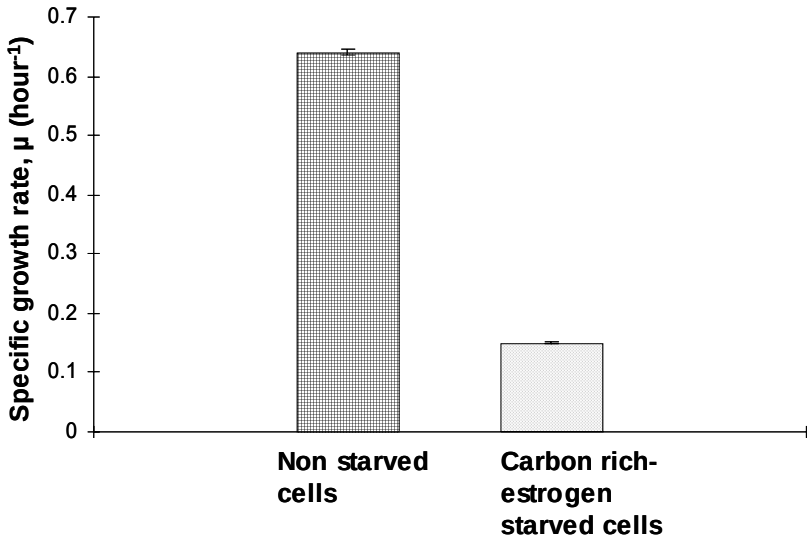


Figure 4.7 **Effects of nutrient-rich medium on the specific growth rate of 17β-estradiol. The error bars represent the range of repeated experiments.**

estrogen starved cells was calculated from data measured during the first 24 hours (refer to Table C.3 in Appendix C for calculation). For the non-starved cells, the specific rate was calculated from the data observed during the first 3 hours. The 17β-estradiol degradation rate for LB-starved cells was decreased by 75% of that for non-starved cells.

4.3.2 Effects of Complex Medium on 17β-estradiol Degradation

Experiments were conducted to test the degradation ability of completely starved cells. ARI-1 cells were starved from 17β-estradiol and any other carbon sources in the NMS medium for 1, 3, and 7 days.

Figures 4.8-4.10 show the biotransformation of 17β-estradiol by 1day-completely starved, 3day-completely starved, and 7day-completely starved ARI-1 cells. As shown in Figure 4.8, the 17β-estradiol concentration was reduced by half in 48hours by 1day-completely starved cells. The depletion of the 17β-estradiol slowed and was followed by the formation of estrone during the monitoring period (200 hours).

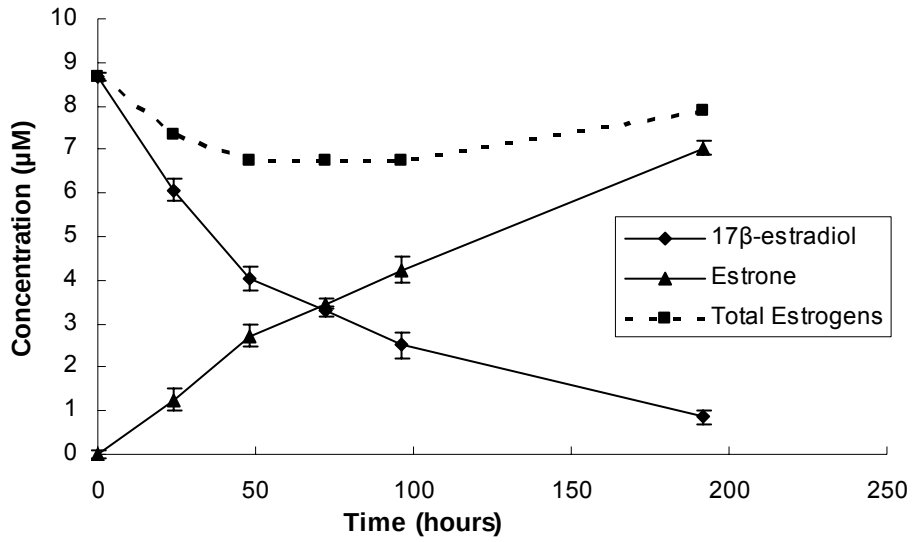


Figure 4.8 **Degradation of 17β-estradiol by 1day-completely starved cells. The error bars represent the range of duplicates.**

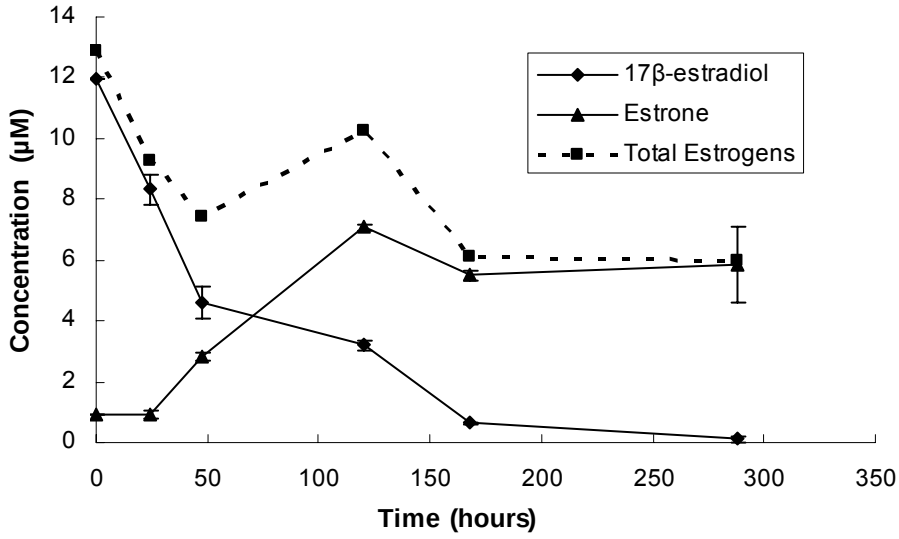


Figure 4.9 **Degradation of 17β-estradiol by 3day-completely starved cells. The error bars represent the range of duplicates.**

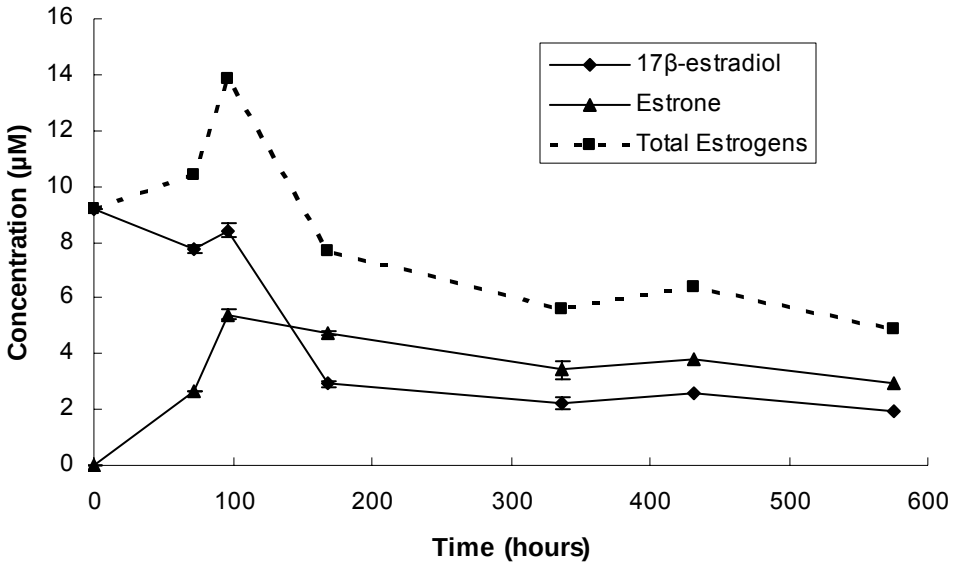


Figure 4.10 **Degradation of 17β-estradiol by 7day-completely starved cells. The error bars represent the range of duplicates.**

Figure 4.9 shows the degradation of 17β-estradiol and estrone exhibited by 3day-starved cells. Degradation of 17β-estradiol was fast in the first 48 hours and then decreased, similar to the case for 1d-starved cells. Although the concentration of estrone increased in the first 125 hours, the concentration began to decline slowly, and leveled off at the end of the experiment.

Figure 4.10 shows the experimental data of 17β-estradiol degradation by 7day-starved cells. In this case, the degradation of 17β-estradiol progressed quickly during the first 168 hours. After 168 hours, the degradation rate decreased.

Some interesting trends were observed from these tests: (1) estrone accumulated during 17β-estradiol degradation by all starved cells (starved from either estrogen alone or from both estrogen and all other carbons), and (2) the concentration of estrone started

to decline, but the degradation rate was much lower compared to non-starved cells. In general, the longer the starvation period, the longer the time it took for degrading the estrogens.

The specific degradation rates of 17β-estradiol for non-starved and completely starved cells were calculated (Figure 4.11). 17β-estradiol degradation rates for 1day-starved and 3day-starved cells were decreased by 90% of that of non-starved cells. The degradation rate for 7day-starved cells decreased to less than 0.1% of that of non-starved cells. Figure 4.12 shows the overall degradation efficiency of 17β-estradiol of non-starved, carbon rich-estrogen starved, and completely starved cells at 168 hours. Carbon rich-estrogen starved cells were able to degrade 15% of total estrogen.

However, 1day-completely starved, 3day-completely starved, and 7day-completely starved cells were able to degrade 22%, 52%, and 10% of total estrogen respectively during the monitoring periods.

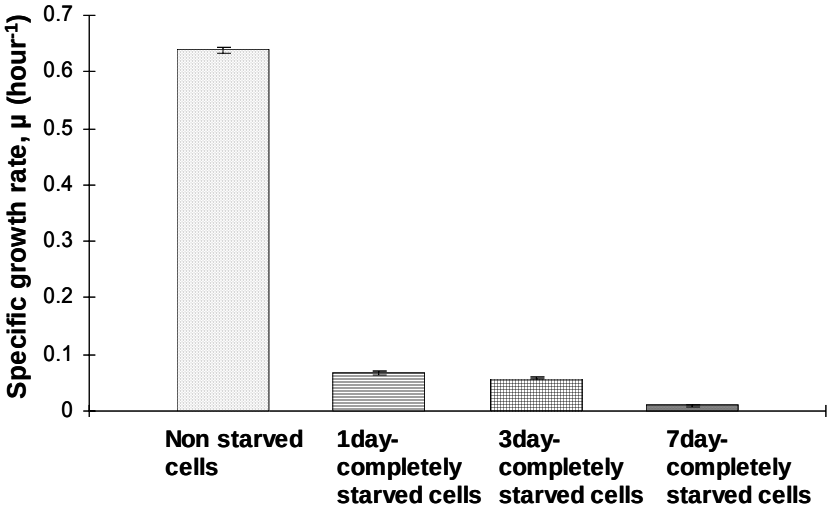


Figure 4.11 **Comparison of specific growth rate of 17β-estradiol degradation of completely starved cells and non-starved cells. The error bars represent the range of three repeated experiments.**

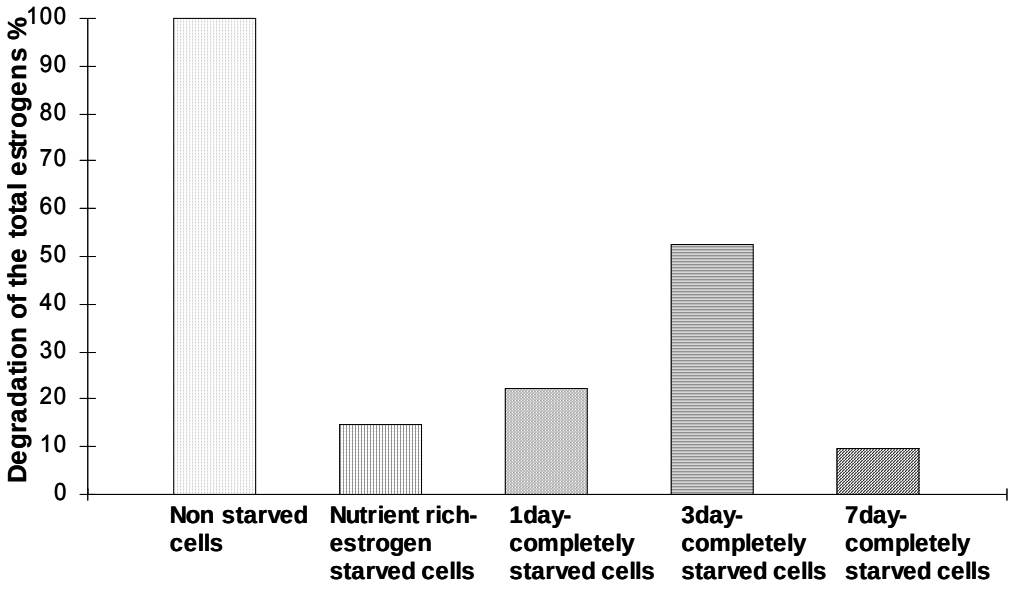


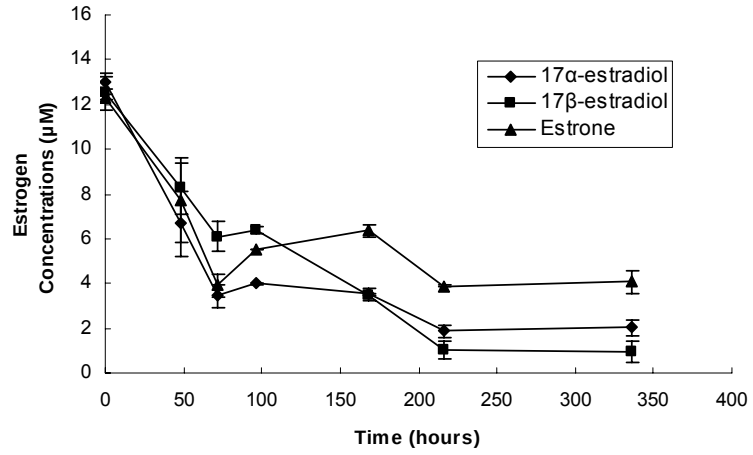
Figure 4.12 **Comparison of % degradation of total estrogen by non-starved, nutrient rich-estrogen starved, and completely starved cells. Total estrogen=17β-estradiol + estrone.**

4.4 Effects of Estrogen Mixtures on Estrogen Degradation

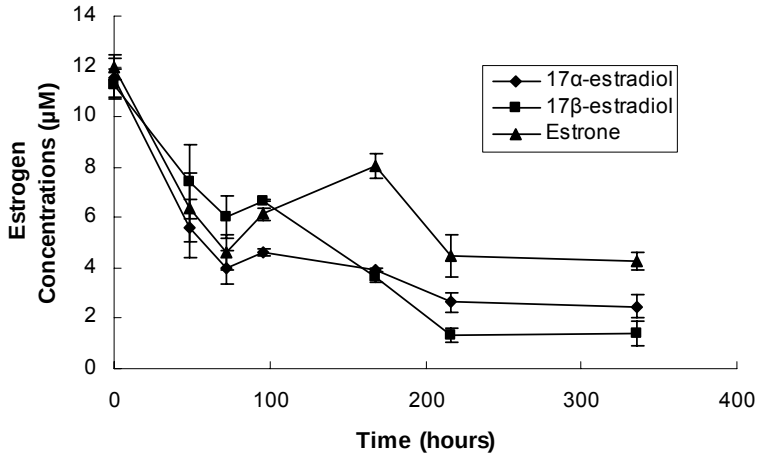
Since estrogen mixtures are commonly detected in wastewater, it is important to know if estrogen mixtures affect the estrogen degradation pattern of ARI-1. The mixed estrogen effect tests were carried out in a manner similar to the growth experiments except that mixtures of the three estrogens (3mg/L each) were added in the NMS medium.

Figure 4.13 (a)-(c) show the results of three sets of repeated experiments using ARI-1 to degrade an estrogen mixture: 17α-estradiol, 17β-estradiol and estrone.

(a)



(b)



(c)

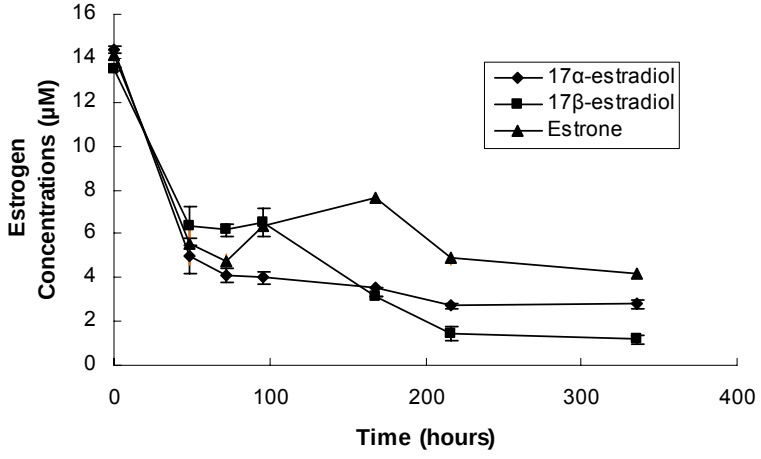


Figure 4.13 **Degradation of an estrogen mixture by ARI-1 in three repeated experiments. The error bars represent the range of duplicates.**

For easy comparison, total estrogen was calculated and plotted in Figure 4.14. Similar degradation trends were observed in three repeated experiments. No significant degradation preference of three three estrogens was observed in the first 48 hours. Estrone concentration slightly increased during 72 hours to 168 hours and then decreased. The increase of estrone concentrations is likely be due to the conversion of 17α-estradiol and 17β-estradiol to estrone.

4.5 Sampling WWTPs in East Tennessee Using Real-time-t-RFLP Assay

Experiments were conducted to examine the ubiquitousness of ARI-1 in WWTPs with varying operation conditions. A novel molecular technique, real-time-t-RFLP, was used to analyze microbial communities of activated sludge samples collected from six WWTPs in Knoxville, Tennessee. Operation conditions of WWTPs, including HRT,

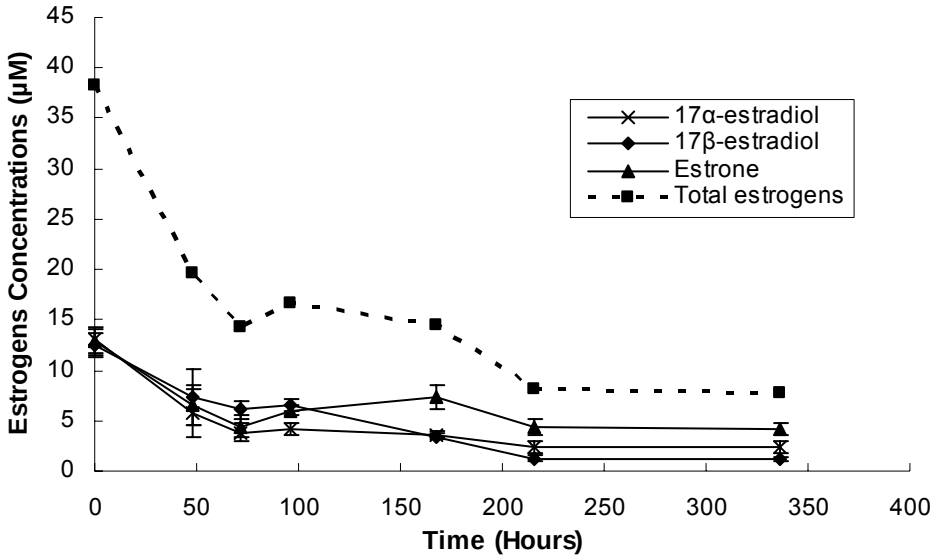


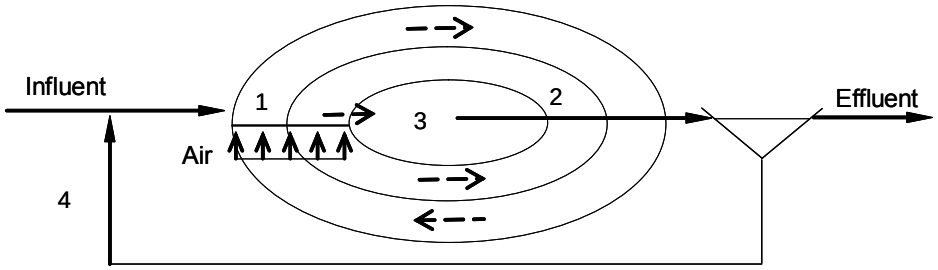
Figure 4.14 **Comparison of estrogen concentrations in the estrogen mixtures effect experiment. The error bars represent the range of three repeated experiments.**

SRT, BOD loading, DO, and TKN are summarized in Table 3.1 (refer to Section 3.2.4).

The biological treatment process used in WWTP1 is an oxidation ditch. Along the oxidation ditch, nutrient and dissolved oxygen concentration change. Accordingly, microbial community structure (types and quantities of microorganisms) changes in response to the changes in nutrient and dissolved oxygen concentrations. Activated sludge samples were collected from four different locations: at the entrance, in the middle, at the exit, and from the return sludge site (Figure 4.15). Figure 4.15 shows the profiles of microbial communities of four samples collected in WWTP1 as characterized by the frequency distribution of DNA fragment sizes after cutting with *Msp1*. The dominant fragment sizes were 83bp, 89bp, 103bp, 105bp, 328bp, and 329bp with DNA copies of 1.3×10^9/mL, 3.4×10^9/mL, 6.8×10^9/mL, 4.2×10^9/mL, 2.6×10^9/mL, and 1.3×10^9/mL at site1 respectively. As shown in the Figure 4.15, the DNA fragment size distribution (types and abundance) change along with the oxidation ditch.

WWTP2 uses the plug-flow aeration tank in the activated sludge system, with even aeration along the tank. Figure 4.16 shows the real-time-t-RFLP calculation results of the five sampling sites in WWTP2. As shown in this figure, the DNA copies increase from sampling site 1 to sampling site 5, which is in the direction of wastewater flow. The five sampling sites in this test show similar microbial community profiles, the dominant fragment sizes were 83bp, 98bp, 102bp, 103bp, 105bp, and 328bp with higher DNA copies of 2.3×10^9/mL, 2.3×10^9/mL, 2.7×10^9/mL, 2.8×10^9/mL, 3.3×10^9/mL, and 5.3×10^9/mL respectively.

WWTP3, WWTP4, WWTP5, and WWTP6 use a CSTR aeration tank in the activated sludge system. Figure 4.17 shows the real-time-t-RFLP results of samples collected from



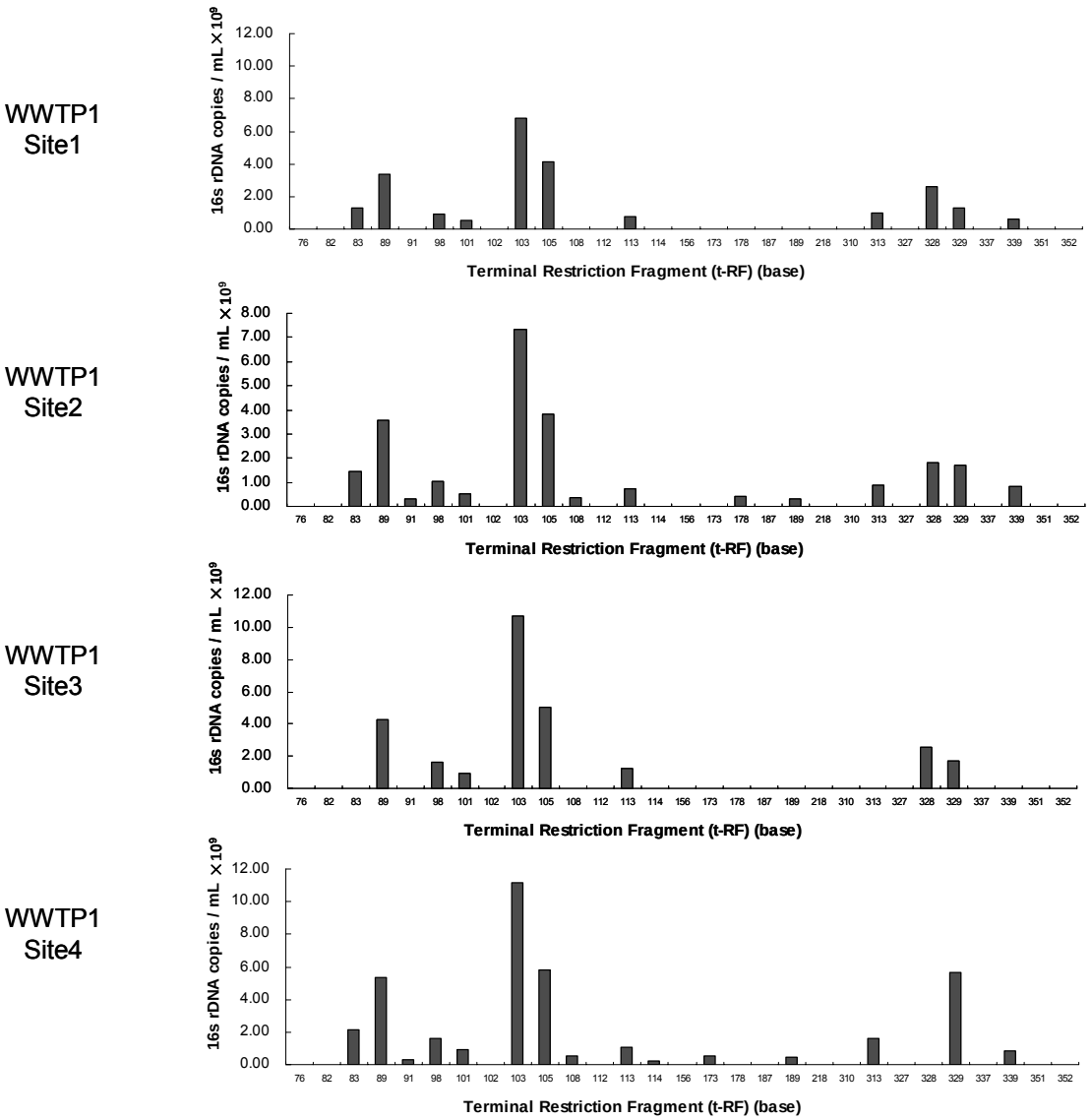


Figure 4.15 **Real-time-t-RFLP analysis for activated sludge samples collected from WWTP1.**

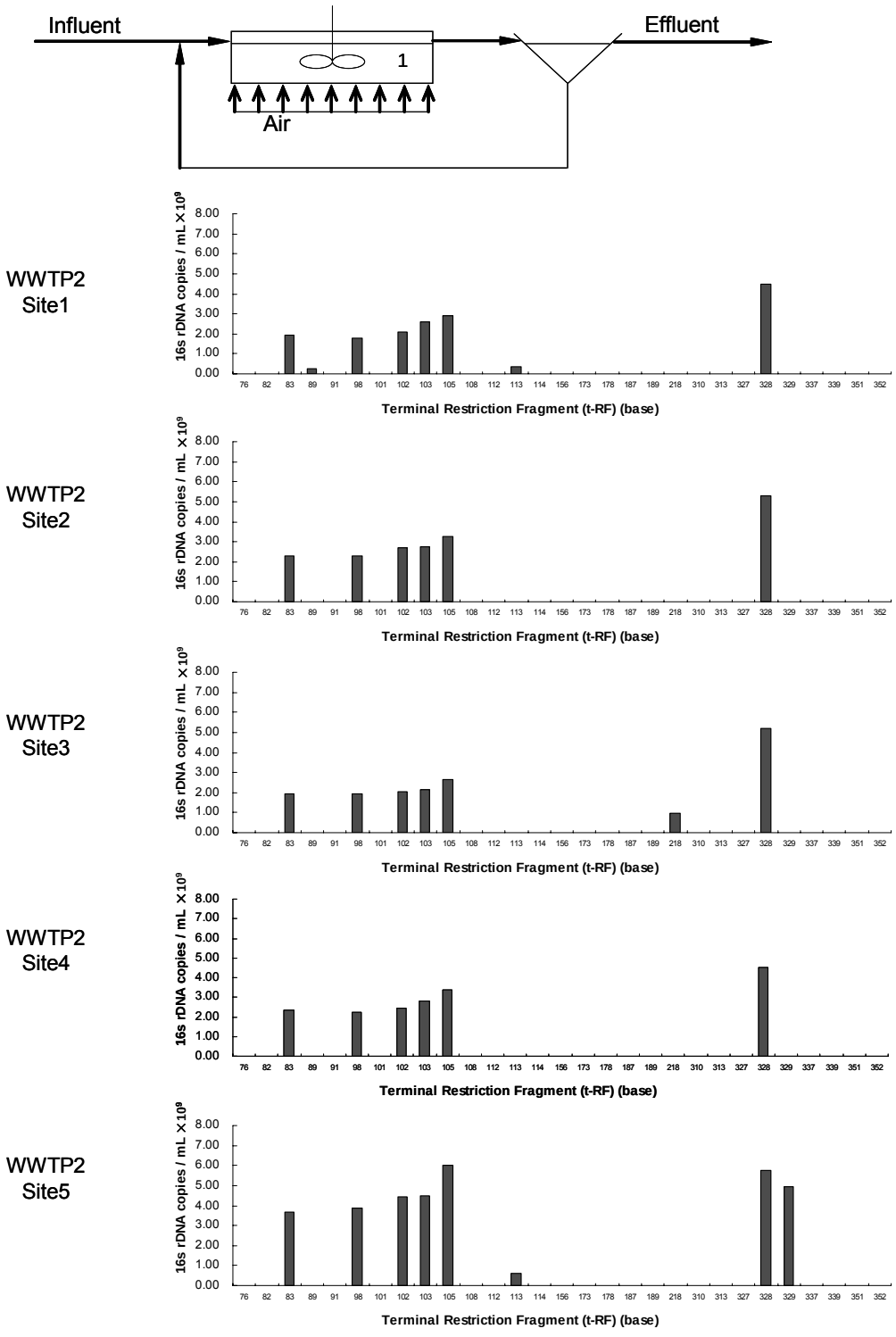


Figure 4.16 **Real-time-t-RFLP analysis for activated sludge samples collected from WWTP2.**

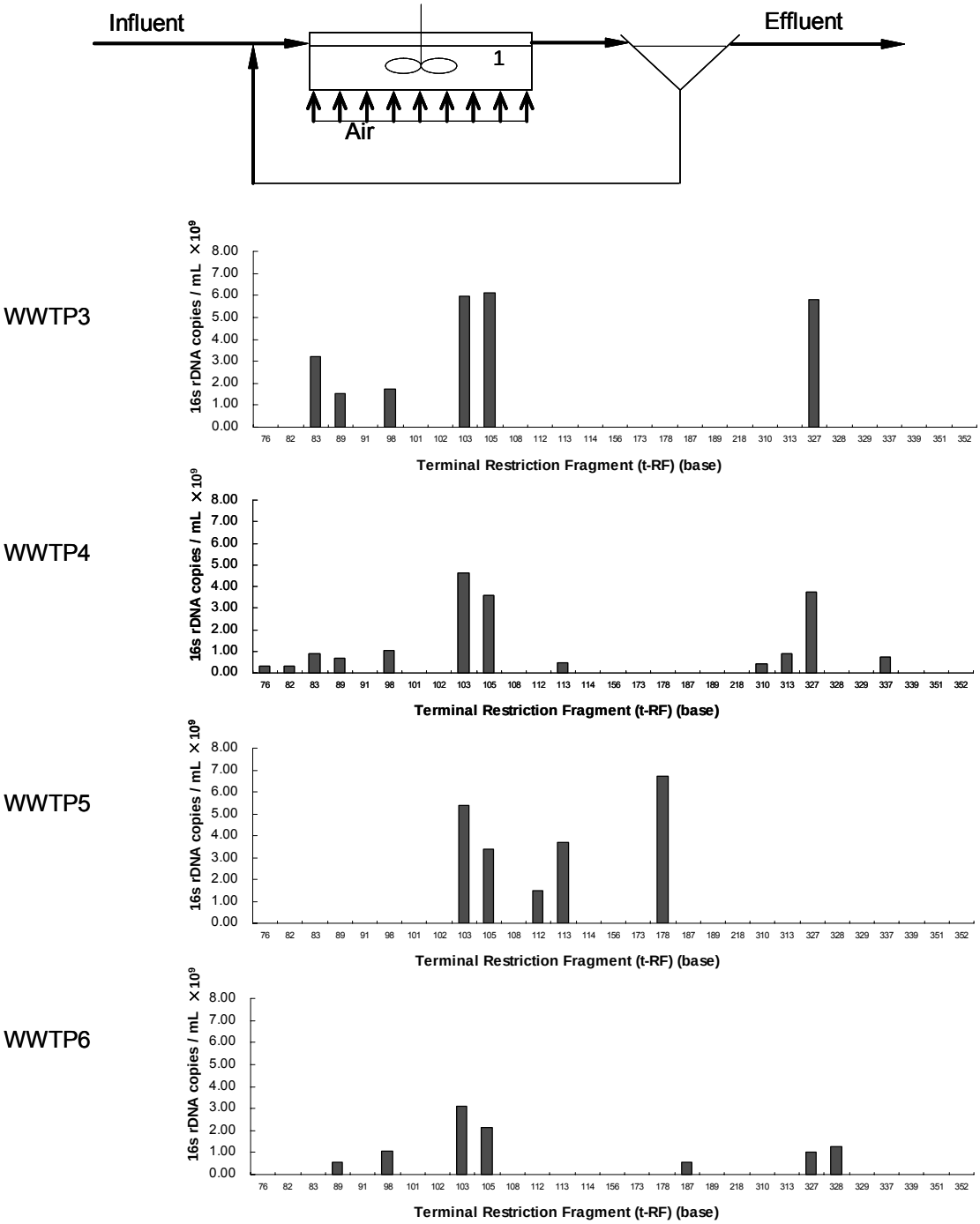


Figure 4.17 **Real-time-t-RFLP analysis for activated sludge samples collected from WWTP3, WWTP4, WWTP5, and WWTP6.**

these WWTPs. For WWTP3, the dominant fragment sizes were 83bp, 89bp, 98bp 103bp, 105bp, and 327bp with DNA copies of 3.2×10^9/mL, 1.5×10^9/mL, 1.7×10^9/mL, 6.0×10^9/mL, 6.1×10^9/mL, and 5.8×10^9/mL respectively; for the WWTP4, the dominant fragment sizes were 83bp, 89bp 98bp, 103bp, 105bp, 313bp, 327bp, and 337bp with DNA copies of 9.0×10^8/mL, 6.8×10^8/mL, 1.1×10^9/mL, 4.6×10^9/mL, 3.6×10^9/mL, 8.8×10^8/mL, 3.8×10^9/mL, and 7.5×10^8/mL; for the WWTP5, the dominant fragment sizes were 103bp, 105bp, 112bp, 113bp, and 178bp with the total DNA copies of 5.4×10^9/mL, 3.4×10^9/mL, 1.5×10^9/mL, 3.7×10^9/mL, and 6.7×10^9/mL separately; for the WWTP6, the dominant fragment sizes were 89bp, 98bp, 103bp, 105bp, 187bp, 327bp and 328bp, for which the DNA copies are 5.6×10^8/mL, 1.1×10^9/mL, 3.1×10^9/mL, 2.1×10^9/mL, 5.7×10^8/mL, 1.0×10^9/mL, and 1.3×10^9/mL.

Figure 4.18 shows the fragment length (a size of 103bp) corresponding to a pure ARI-1 culture. A fragment size of 103bp was observed in all sludge samples surveyed, suggesting possibility of the presence of ARI-1. However, no further experiments were conducted to provide positive identification of ARI-1.

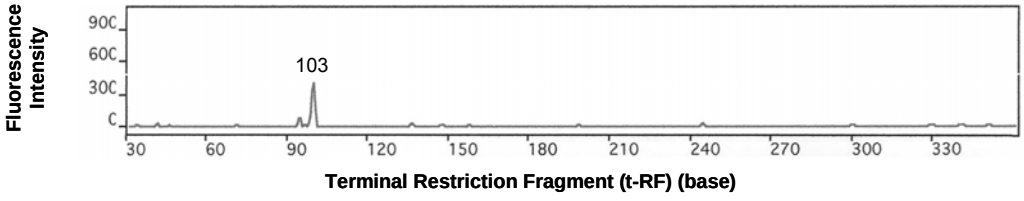


Figure 4.18 **t-RFLP experiment results for ARI-1 pure culture.**

CHAPTER FIVE

DISCUSSIONS

The growth yields (Y, mg VSS/mg estrogen) of ARI-1 for 17β-estradiol and estrone were Y=0.08±0.01mg VSS/mg 17β-estradiol and Y=0.05±0.01mg VSS/mg estrone. These values were much smaller than those observed for other heterotrophic bacteria grown on wastewater (a typical value is Y=0.45mg VSS/mg BOD; refer to Table 2.1 for details), suggesting that estrogens are poor growth substrates for ARI-1.

Although the measured $\hat{q}$ and K_m are comparable to those observed in activated sludge, the small growth yield and desired effluent concentrations (in ng/L) suggested that using ARI-1 to degrade estrogen in wastewater is challenging. If a WWTP influent contains 200ng/L of 17β-estradiol and the desired effluent concentration of 17β-estradiol is 10ng/L, then based on K_m=14.7mg BOD_5/L and μ_m=1.9 mg VSS/mg BOD_5-day in the kinetic experiments (refer to Table 4.2 for details), μ would be 2.6×10^{-5} mg VSS/mg BOD_5-day. The required solid retention time ($\theta=1/\mu$) will be 38460 days, which is 1923 times higher than a typical SRT (20 days) for an activated sludge system.

ARI-1 was capable of degrading estrogens when grown with estrogens and acetone, suggesting that the presence of 17β-estradiol in growth medium is required for ARI-1 to retain the ability to degrade estrogens. This was supported by the results of effects of starvation experiments (Figure 4.5-4.11).

Results of experiments using carbon rich-estrogen starved and completely starved cells are interesting. For carbon rich-estrogen starved cells, ARI-1 lost the ability to

degrade estrone produced from 17β-estradiol degradation. However, after the complete absence of any carbon source, ARI-1 cells were still capable of degrading 17β-estradiol and estrone (refer to Figure 4.11 for details) although at much slower rates than cells that were constantly exposed to estrogens. These results suggest that enhanced 17β-estradiol degradation might be possible by growing ARI-1 with other carbon sources in the presence of estrogens (to prevent the lost of estrogen-degrading ability) in bioreactors. For the carbon rich-estrogen starved cells, the incomplete degradation of 17β-estradiol to estrone was disappointing. However, the total estrogenicity of samples decreased dramatically. These findings might explain the high estrone concentrations and low 17β-estradiol concentrations in the effluent of WWTPs surveyed (Johnson and Sumpter 2002; Anderson et al., 2003).

The fragment size of 103bp (similar to the measured fragment length for ARI-1) observed in Figure 4.15-4.17 suggested the presence of ARI-1 in the activated sludge system. However, since many strains are known to contribute the fragment of 103bp, more detailed molecular analysis, such as clone library or FISH, is needed to confirm the presence of ARI-1 in the activated sludge surveyed.

Despite the fact that estrogens in our aquatic environment have been recognized as an emerging problem, little research has been focused on the biodegradation of estrogens in wastewater (Fujii et al., 2002; Layton et al., 2002; Anderson et al., 2003). The results of this study, including the kinetic parameters of estrogen degradation by ARI-1, studies of the starvation effects, and the suspected prevalence of ARI-1 in various activated sludge systems, are a useful starting point for more detailed studies of how estrogen removal can be enhanced in WWTPs.

CHAPTER SIX

SUMMARY, CONCLUSIONS, AND FUTURE STUDIES

6.1 Summary

In this study, a series of experiments were conducted to characterize ARI-1's ability to degrade estrogens. Effects of acetone on the growth of ARI-1 using three different estrogens were examined. Degradation kinetic parameters, $\hat{q}$ and K_m for 17β-estradiol and estrone, were measured. In addition, the effects of carbon rich-estrogen starvation and completely starvation on estrogen degradation were elucidated. Experiments were also conducted to test the ability of ARI-1 to degrade a mixture of three estrogen mixtures. Finally, a quantitative fingerprinting molecular method was used to examine the prevalence of DNA restriction fragments characteristic of ARI-1 in activated sludge system of six WWTPs in Knoxville, Tennessee.

6.2 Conclusions

The following conclusions can be drawn from this study:

(1) ARI-1 degrades estrone faster than 17β-estradiol, all other conditions being equal;

(2) ARI-1 retains its estrogen-degrading ability as long as estrogens are present in its environment;

(3) Lack of an estrogen source for several days will significantly decrease ARI-1's estrogen degradation ability;

(4) ARI-1 is capable of lowering the total estrogenicity in its environment by transforming 17β-estradiol to estrone even if its estrogen-degrading ability is compromised;

(5) ARI-1 shows no much preference for estrogen degradation in mixtures of estrogens.

6.3 Future Studies

All experiments were conducted in a batch system in this study. From an engineering perspective, a controlled scale-chemostat operated under various conditions to mimic real WWTPs will be particularly useful in studying the role and the significance of ARI-1 in estrogen degradation.

Further, studies focused on the elucidating degradation pathways of 17α-estradiol, 17β-estradiol, and estrone by ARI-1 and identifying and characterizing the enzymes responsible for estrogen degradation are needed. Estrogen is aerobically degraded by ARI-1 (Fujii et al., 2002) suggesting that oxygenase might be a key enzyme for estrogen degradation. Experiments designed for determine the presence and types of oxygenases will be particularly useful in understanding degradation pathway of estrogens by ARI-1.

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APPENDIXES

APPENDIX A: Supplementary Information for Estrogens

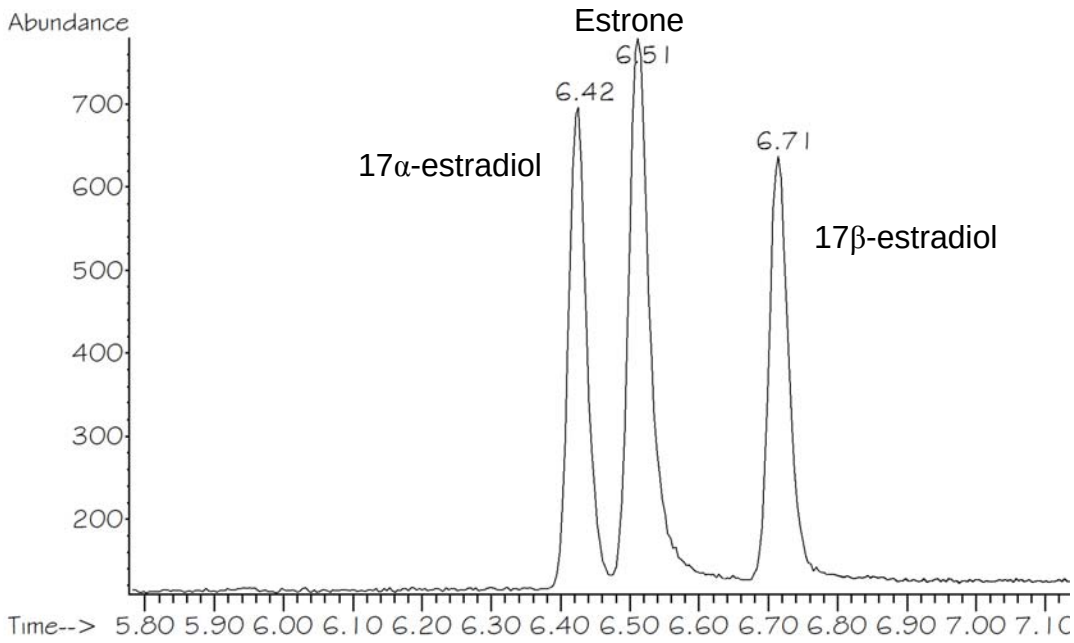

Figure A.1 **Typical retention time for 17α-estradiol, estrone, and 17β-estradiol.**

Calculation of COD for 17β-estradiol and estrone:

$C_{18}H_{24}O_2$ (17β-estradiol) + $23O_2 \rightarrow 18CO_2 + 12H_2O$

For 3mg/L of 17β-estradiol, the COD=3mg/L× (23×32)/272=8.1mg/L

$4C_{18}H_{23}O_2$ (estrone) + $91O_2 \rightarrow 72CO_2 + 46H_2O$

For 3mg/L of estrone, the COD=3mg/L× (91×32)/ (271×4)=8.1mg/L

APPENDIX B: Supplementary Information and Experimental Data for Growth/Kinetic Experiments

Table B.1 **Comparison of linear-regression plots results**

	Kinetic Parameters	Lineweaver-Burk	Eadie-Hofstee	Hanes-Woolf
17β-estradiol	Km (mg/L)	7.8	10.4	17.3
	$\hat{q}$ (mg / mgVSS-day)	14.0	19.5	26.0
estrone	Km (mg/L)	5.1	3.7	6.4
	$\hat{q}$ (mg / mgVSS-day)	14.0	12.3	16.9

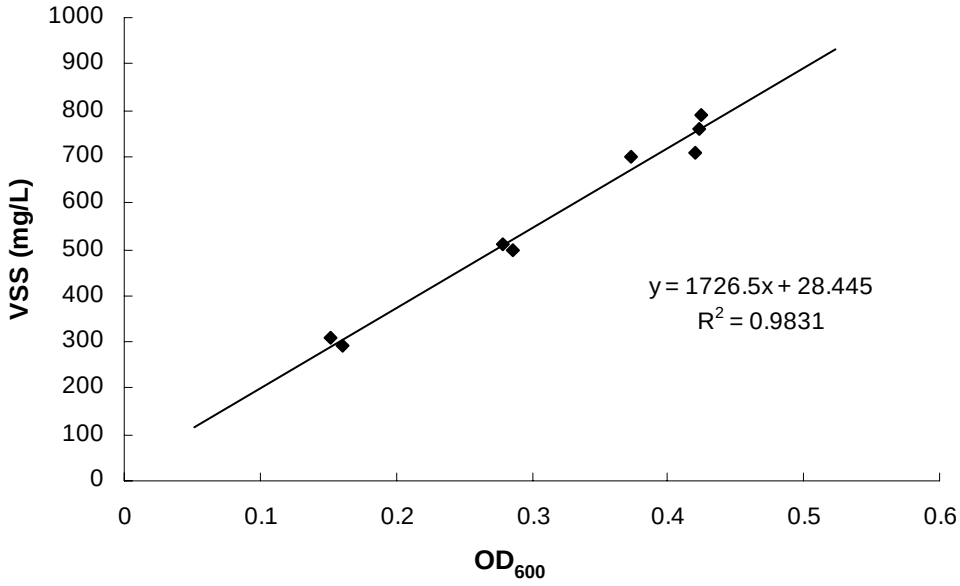

Figure B.1 **Correlation of OD_{600} and VSS.**
VSS (mg/L)= 1726.5×OD600+28.445.

Table B.2 **Correlation data of OD_{600} and dry cell mass as VSS for Figure B.1**

	Experiment Sample	OD_{600}	Weigh1(g)	Weigh2 (g)	Biomass(g)	Biomass Concentration, VSS (mg/L)
1	17	0.1512	1.0193	1.0162	0.0031	310
	18	0.1602	1.3634	1.3605	0.0029	290
2	17	0.2784	1.3746	1.3695	0.0051	510
	18	0.2858	1.3760	1.3710	0.0050	500
3	17	0.4198	1.3825	1.3754	0.0071	710
	18	0.3726	1.3694	1.3624	0.0070	700
4	17	0.4240	1.0327	1.0248	0.0079	790
	18	0.4231	1.3771	1.3695	0.0076	760

Table B.3 **Growth w/ and w/o acetone experiment data (Figure 4.1 and Figure 4.2)**

Exp. Samples	OD_{600}							
	0 hour	24 hours	36 hours	48 hours	60 hours	72 hours	84 hours	96 hours
[1] C1	0.0057	0.0051	0.0051	0.0051	0.0054	0.0048	0.0092	0.0050
[1] C2	0.0061	0.0053	0.0051	0.0054	0.0055	0.0052	0.0070	0.0053
[2] AW3	0.0110	0.0571	0.1838	0.3550	0.2843	0.2616	0.2416	0.2281
[2] AW4	0.0113	0.0570	0.1851	0.3676	0.2857	0.2641	0.2477	0.2400
[3] AWO5	0.0115	0.0689	0.1936	0.1831	0.1483	0.1549	0.1432	0.1426
[3] AWO6	0.0114	0.0611	0.2004	0.1699	0.1597	0.1534	0.1600	0.1493
[4] BW7	0.0096	0.0457	0.1494	0.3358	0.2480	0.2424	0.2352	0.2224
[4] BW8	0.0099	0.0485	0.1549	0.3311	0.2707	0.2289	0.2325	0.2251
[5] BWO9	0.0103	0.0552	0.1232	0.1113	0.1022	0.0811	0.0772	0.0708
[5]BWO10	0.0103	0.0610	0.1318	0.1019	0.0874	0.0871	0.0655	0.0893
[6] EW11	0.0111	0.0541	0.1443	0.2522	0.2476	0.2485	0.2397	0.2613
[6] EW12	0.0124	0.0550	0.1481	0.2491	0.2550	0.2507	0.2586	0.2576
[7] EWO13	0.0099	0.0766	0.0649	0.0608	0.0429	0.0450	0.0429	0.0426
[7] EWO14	0.0100	0.0832	0.0668	0.0539	0.0406	0.0419	0.0546	0.0368

[1] data for blank control; [2] data for 3mg/L of 17α-estradiol with acetone; [3] data for 3mg/L of 17α-estradiol without acetone; [4] data for 3mg/L of 17β-estradiol with acetone; [5] data for 3mg/L of 17β-estradiol without acetone; [6] data for 3mg/L of estrone with acetone; [7] data for 3mg/L of estrone without acetone

Table B.4 **Experimental data and calculation for Figure 4.3**

No.	17β-estradiol Concentration (μg/L)		OD_{600} [1]		q ($hour^{-1}$) [2]	S (μg/L) [3]	1/q	1/S
	0hour	5 hours	0hour	5hour				
1	4159	2949	0.5477	0.5564	0.4429	4159	2.2581	0.0002
2	1928	1711	0.5477	0.5564	0.1000	1928	9.9959	0.0005
3	367	331	0.5477	ND [4]	0.0226	367	44.3089	0.0027
4	401	369	0.5477	ND [4]	0.0218	401	45.9767	0.0025
5	294	266	0.5477	ND [4]	0.0210	294	47.5682	0.0034
6	284	251	0.5477	ND [4]	0.0219	284	45.7376	0.0035

[1] the correlation of X_{VSS} and OD600: $X_{VSS}=1726.5\times OD_{600}+28.445$, mg/L.

[2] $q=\frac{1}{X}\frac{dS}{dt}$ ($X=X_0$, mg/L; $dS=S_0-S_5$; dt=5 hours)

[3] $S=S_0$

[4] ND: not determined

***Table B.5* Experimental data and calculation for Figure 4.4**

No.	Estrone Concentration (μg/L)		OD_{600} [1]		q ($hour^{-1}$) [2]	S (μg/L) [3]	1/q	1/S
	0hour	5 hours	0hour	5hour				
1	9587	8544	1.0203	0.9976	0.2241	9587	4.4626	0.000104
2	9690	6629	1.0203	1.0511	0.4496	9690	2.2241	0.000103
3	5666	4380	1.0203	ND [4]	0.2513	5666	3.9794	0.000176
4	4802	4094	1.0203	ND [4]	0.1868	4802	5.3542	0.000208
5	877	126	1.0203	ND [4]	0.0923	877	10.8364	0.00114
6	732	221	1.0203	ND [4]	0.0654	732	15.2812	0.001365
7	302	114	1.0203	ND [4]	0.0293	302	34.1526	0.003313
8	177	83	1.0203	ND [4]	0.0189	177	52.8801	0.005635

[1] the correlation of X_{VSS} and OD600: $X_{VSS}=1726.5\times OD_{600}+28.445$, mg/L.

[2] $q = \frac{1}{X}\frac{dS}{dt}$ ($X=X_0$, mg/L; $dS=S_0-S_5$; dt=5 hours)

[3] $S=S_0$

[4] ND: not determined

APPENDIX C: Supplementary Information and Experiment Data for Starvation Effects and Estrogen Mixtures Effects

Table C.1 **Experimental data and calculation for Figure 4.5**

	0 hour		24 hours		48 hours	
	17β-estradiol Concentration (mM)	Estrone Concentration (mM)	17β-estradiol Concentration (mM)	Estrone Concentration (mM)	17β-estradiol Concentration (mM)	Estrone Concentration (mM)
1	10.01	-	3.42	7.72	-	9.33
2	9.59	0.16	3.10	7.39	-	10.00
3	10.72	-	2.63	62.57	-	8.86
4	11.26	-	2.74	48.64	-	9.56
5	10.42	-	3.00	72.46	-	6.69
6	12.04	0.10	2.69	7.73	-	7.28

Table C.1 **Continued**

	288 hours	
	17β-estradiol Concentration (mM)	Estrone Concentration (mM)
1	-	9.31
2	-	9.80
3	-	8.69
4	-	8.49
5	-	8.86
6	-	9.94

***Table C.2* Data for Figure 4.6**

		17β-estradiol Concentration (mM)	Estrone Concentration (mM)
0 hour	1	10.04	0.36
	2	10.28	0.34
3 hours	3	4.24	1.24
	4	5.00	1.57
6 hours	5	1.16	0.91
	6	1.38	0.71
9 hours	7	0.00	0.00
	8	0.00	0.00

***Table C.3* Experimental data and calculation for Figure 4.7**

	No.		Concentration (μg/L)		dS (μg/L)	OD_{600} (0 hour)	VSS(mg/L)	μ(hr^{-1})[1]	Average	Range
			0 hour	24 hours						
LB	17β-estradiol	1	2989	731	2258	0.3512	634.8018	0.148209	0.149954	0.001745
		2	3055	774	2281	0.3464	626.5146	0.151699		
	Estrone	3	22	2054	2032	0.3512	634.8018	0.133375	0.133958	0.000583
		4	13	2036	2023	0.3464	626.5146	0.134541		
	No.		Concentration (μg/L)		dS (μg/L)	OD_{600} (0 hour)	VSS(mg/L)	μ(hr^{-1})	Average	Range
			0 hour	6 hours						
Non	Estrone		2731	314	2417	0.3512	634.8018	0.634581	0.639576	0.004995
		2	2796	373	2423	0.3464	626.5146	0.644571		
		3	98	337	239	0.3512	634.8018	0.125498	0.151867	0.026368
		4	93	428	335	0.3464	626.5146	0.178235		

[1] $\mu = Y \times (dS)/(VSS \times dt)$. where Y is the yields.

Table C.4 **Experimental data and calculation for Figure 4.8**

	0 hour		24 hours		48 hours	
	17β-estradiol Concentration (mM)	Estrone Concentration (mM)	17β-estradiol Concentration (mM)	Estrone Concentration (mM)	17β-estradiol Concentration (mM)	Estrone Concentration (mM)
1	7.99	-	6.42	1.31	3.88	1.69
2	9.50	-	6.21	1.53	3.68	3.52
3	7.32	-	5.44	1.46	3.21	2.99
4	9.85	-	6.24	1.33	5.40	3.12
5	7.75	-	5.47	1.08	4.58	2.31
6	8.07	-	5.34	1.14	4.89	2.43
	72 hours		96 hours		192 hours	
	17β-estradiol Concentration (mM)	Estrone Concentration (mM)	17β-estradiol Concentration (mM)	Estrone Concentration (mM)	17β-estradiol Concentration (mM)	Estrone Concentration (mM)
1	3.53	2.56	3.04	5.08	1.49	6.94
2	2.80	3.82	2.57	7.53	0.56	7.52
3	2.68	4.03	1.48	3.62	0.51	7.29
4	4.11	3.51	2.97	4.10	0.88	9.10
5	4.04	2.95	3.64	4.35	1.24	5.50
6	4.67	3.26	4.16	4.82	1.46	6.11

Table C.5 **Experimental data and calculation for Figure 4.9**

	0 hour		24 hours		48 hours	
	17β-estradiol Concentration (mM)	Estrone Concentration (mM)	17β-estradiol Concentration (mM)	Estrone Concentration (mM)	17β-estradiol Concentration (mM)	Estrone Concentration (mM)
1	13.69	1.23	7.88	1.38	4.18	1.86
2	10.24	0.57	7.81	0.74	3.96	4.10
3	-	-	9.26	0.81	3.44	3.45
4	-	-	9.55	0.86	5.90	3.60
5	-	-	9.77	0.80	4.98	2.61
6	-	-	7.85	0.73	5.33	2.76
	120 hours		168 hours		288 hours	
	17β-estradiol Concentration (mM)	Estrone Concentration (mM)	17β-estradiol Concentration (mM)	Estrone Concentration (mM)	17β-estradiol Concentration (mM)	Estrone Concentration (mM)
1	3.52	6.24	0.57	-	0.21	-
2	3.21	8.07	0.74	5.65	0.23	7.07
3	2.81	5.42	0.51	-	0.15	-
4	3.42	8.61	0.61	4.57	0.03	4.94
5	2.81	5.28	0.53	-	0.03	-
6	3.30	8.58	0.67	5.33	0.03	4.57

***Table C.6* Experimental data and calculation for Figure 4.10**

	0 hour		48 hours		72 hours		96 hours	
	17β-estradiol Concentration (mM)	Estrone Concentration (mM)	17β-estradiol Concentration (mM)	Estrone Concentration (mM)	17β-estradiol Concentration (mM)	Estrone Concentration (mM)	17β-estradiol Concentration (mM)	Estrone Concentration (mM)
1	10.49	0.70	7.72	1.07	8.19	3.19	7.51	4.65
2	7.90	0.38	5.89	0.69	7.01	2.52	7.35	5.00
3	10.49	-	6.26	0.96	8.43	3.05	8.83	5.25
4	7.90	-	4.40	0.66	6.81	2.27	8.53	5.90
5	10.49	-	5.94	0.91	9.07	2.97	8.81	5.36
6	7.90	-	5.05	0.59	6.76	2.28	7.52	5.07
	168 hours		336 hours		432 hours		576 hours	
	17β-estradiol Concentration (mM)	Estrone Concentration (mM)	17β-estradiol Concentration (mM)	Estrone Concentration (mM)	17β-estradiol Concentration (mM)	Estrone Concentration (mM)	17β-estradiol Concentration (mM)	Estrone Concentration (mM)
1	2.24	3.42	2.25	4.32	2.22	4.13	1.50	2.71
2	2.68	4.66	1.97	2.27	2.96	2.82	2.62	3.21
3	2.74	3.93	2.19	4.11	2.08	4.01	1.63	2.78
4	3.31	5.64	2.65	3.29	3.01	2.76	2.49	3.39
5	2.53	3.94	2.00	3.79	2.49	5.73	1.38	2.84
6	3.11	5.33	1.96	2.42	2.77	2.57	2.28	2.65

Table C.7 **Experimental data and calculation for Figure 4.11**

	No.		Concentration (μg/L)		dS (μg/L)	OD_{600}	VSS(mg/L)	μ(hr^{-1})	Average	Range
			0 hour	6 hours						
Non Starvation	17β-estradiol	1	2731	314	2417	0.3512	634.8018	0.634581	0.639576	0.004995
		2	2796	373	2423	0.3464	626.5146	0.644571		
	Estrone	3	98	337	239	0.3512	634.8018	0.125498	0.151867	0.026368
		4	93	428	335	0.3464	626.5146	0.178235		
	No.		Concentration (μg/L)		dS (μg/L)	OD_{600}	VSS(mg/L)	μ(hr^{-1})	Average	Range
			0 hour	48 hours						
1 Day Starvation	17β-estradiol	1	2379	1027	1352	0.2176	404.1414	0.069695	0.066442	0.003253
		2	2335	1171	1164	0.2058	383.7687	0.063189		
	Estrone	3	0	830	830	0.3676	663.1164	0.026076	0.024068	0.002009
		4	0	644	644	0.3358	608.2137	0.022059		

Table C.7 **Continued**

	No.		Concentration (µg/L)		dS (µg/L)	OD_{600}	VSS(mg/L)	µ(hr-1)	Average	Range
			0 hour	48 hours						
3 Days Starvation	17β-estradiol	1	3254	1107	2147	0.425	762.2175	0.058683	0.058171	0.000512
		2	3254	1402	1852	0.3711	669.1592	0.057659		
	Estrone	3	245	809	564	0.355	641.3625	0.01832	0.017579	0.000741
		4	245	730	485	0.3311	600.0992	0.016837		
	No.		Concentration (µg/L)		dS (µg/L)	OD_{600}	VSS(mg/L)	µ(hr-1)	Average	Range
			0 hour	72 hours						
7 Days Starvation	17β-estradiol	1	2501	2072	429	0.301	548.1315	0.01087	0.009371	0.001499
		2	2501	2152	349	0.3402	615.8103	0.007871		
	Estrone	3	0	723	723	0.301	548.1315	0.01832	0.0172	0.001119
		4	0	713	713	0.3402	615.8103	0.016081		

***Table C.8* Data calculation for Figure 4.12**

	Concentration (μg/L)		% degraded [1]	Ave %	Range
	0 hour	48 hours			
LB	2990	2599	13.1	14.9	1.5
	3055	2556	16.3		
1d-starved	2379	1813	23.8	22.2	1.5
	2336	1852	20.7		
3d-starved	3500	1717	50.9	52.4	1.5
	3499	1614	53.9		
7d-starved	2501	2339	6.5	9.6	3.1
	2501	2186	12.6		

[1] % degraded=1-S_0/S_{48}

***Table C.9* Data calculation for Figure 4.14**

		Estrogen Concentrations (μg/L)						
		0 hours	48 hours	72 hours	96 hours	168 hours	216 hours	336 hours
17α-estradiol	Average	13.00	5.75	3.84	4.20	3.67	2.40	2.43
	Range	1.23	2.36	0.94	0.58	0.30	0.62	0.57
17β-estradiol	Average	12.45	7.35	6.09	6.50	3.42	1.28	1.18
	Range	1.16	2.81	0.92	0.63	0.33	0.34	0.19
Estrone	Average	12.77	6.54	4.42	5.99	7.32	4.39	4.17
	Range	1.33	1.99	1.01	0.50	1.19	0.77	0.60
Total estrogens	Average	38.22	19.63	16.69	14.40	8.06	8.07	7.78

Table C.10 **Experimental data for Figure 4.13**

		Estrogen Concentrations (mM)						
		0 hour	48 hours	72 hours	96 hours	168 hours	216 hours	336 hours
17α-estradiol	1	13.42	8.11	3.97	3.96	3.27	1.55	1.68
	2	12.67	5.24	2.89	4.03	3.80	2.17	2.36
	3	10.81	6.75	4.66	4.78	3.88	2.24	2.02
	4	12.30	4.44	3.33	4.47	3.97	3.01	2.94
	5	14.57	5.79	4.42	4.23	3.53	2.80	2.57
	6	14.23	4.17	3.76	3.73	3.53	2.60	3.00
17β-estradiol	1	13.06	10.15	6.99	6.29	3.09	0.94	0.90
	2	11.99	6.36	5.17	6.41	3.86	1.14	1.02
	3	10.70	8.88	6.88	6.69	3.45	1.05	0.92
	4	11.90	5.96	5.19	6.63	3.82	1.60	1.88
	5	13.61	7.22	6.43	7.13	3.16	1.15	1.00
	6	13.43	5.49	5.89	5.87	3.16	1.78	1.37
Estrone	1	12.43	9.57	4.42	5.49	6.04	3.89	3.57
	2	12.15	5.83	3.40	5.53	6.63	3.85	4.58
	3	11.44	7.74	5.30	6.37	7.56	3.62	3.92
	4	12.39	5.00	3.89	5.88	8.51	5.29	4.63
	5	13.90	6.54	5.01	6.51	7.58	5.14	4.17
	6	14.33	4.55	4.47	6.15	7.58	4.58	4.15

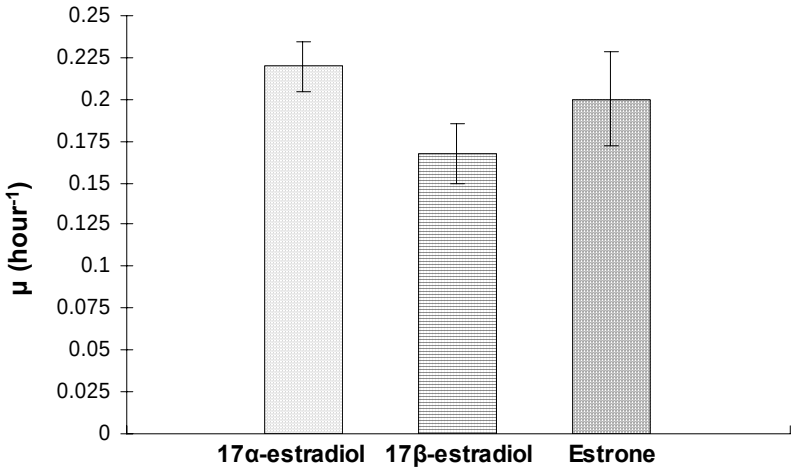

Figure C.1 **Comparison of specific degradation rate of ARI-1 on estrogens mixtures effect experiment. The error bars represent the range of three repeated experiments.**

Table C.11 **Data calculation for Figure C.1**

No.		Concentration (μg/L)		dS (μg/L)	VSS (mg/L)	μ(hr^{-1}) [1]	Average	Range
		0 hour	48 hours					
17α-estradiol	1	3964	1576	2388	242	0.20	0.2199	0.0149
	2	3870	1133	2736	242	0.23		
17β-estradiol	3	3702	1964	1738	242	0.15	0.1673	0.0181
	4	3654	1494	2159	242	0.18		
Estrone	5	3780	1780	1999	242	0.17	0.1999	0.0283
	6	3897	1238	2659	242	0.23		

[1] $\mu = Yq$, refer Table B.4 for q values

APPENDIX D: Supplementary Information of Molecular Techniques and Experimental Data

Calculation for total DNA copies :

$$Total\ DNA\ Copies = \frac{200\mu L \times DNA\ Concentration(ng/\mu L)}{10ng \times 2mL} \times Real\text{-}time\ PCR\ copies$$

where: *200μL* was the total volume of DNA sample;

10ng was the DNA content used for real-time PCR reaction;

2mL was the liquid sample size used for DNA extraction;

Real-time PCR copies are the DNA copies measured in each PCR reaction. The copies were determined from real-time PCR standard curves (refer Figure D.2 in Appendix D for details);

Total DNA Copies were the calculated DNA copies in the original 2mL sludge samples.

Table D.1 **Summary of PCR components**

Components	Descriptions
Thermostable DNA polymerase	*Taq* DNA polymerase from *T. aquaticus*, which belongs to thermophilic Archaea family, is wildly used.
DNA primer	The primer is essential to the PCR reaction. Carefully designed primer would maximize the product yield and suppress amplification of unwanted sequences.
dNTPs	For the standard PCR reactions, it requires equimolar amounts of dATP, dTTP, dCTP, and dGTP.
Divalent cations	Divalent cations, usually Mg^{2+}, are used by thermostable DNA polymerase for activity.
Buffer	Tris-Cl buffer is used for maintaining the pH in the PCR reactions
Template DNA	Single or double stranded DNA containing target sequences

Table D.2 **Summary of DNA concentration after DNA extraction**

WWTP	DNA concentration (μg/μl)	WWTP	DNA concentration (μg/μl)
WWTP5	197	WWTP2, Site1	126
WWTP1, Site1	175	WWTP2, Site2	126
WWTP1, Site2	178	WWTP2, Site3	115
WWTP1, Site3	189	WWTP2, Site4	130
WWTP1, Site4	194	WWTP2, Site5	163
WWTP3	142	WWTP6	180
WWTP4	136		

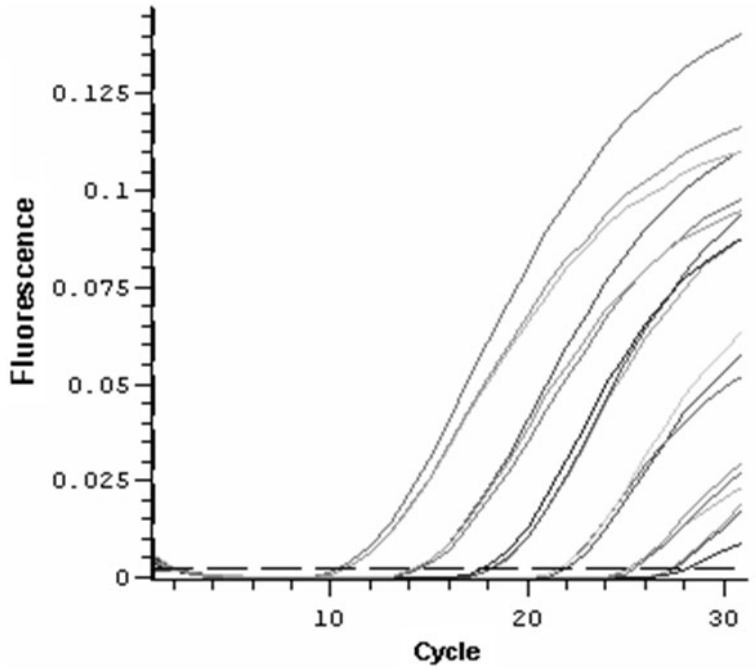

Figure D.1 **Standard curve generated in the Real-time PCR by the cycle dependency of the PCR amplification expressed as the fluorescence.**

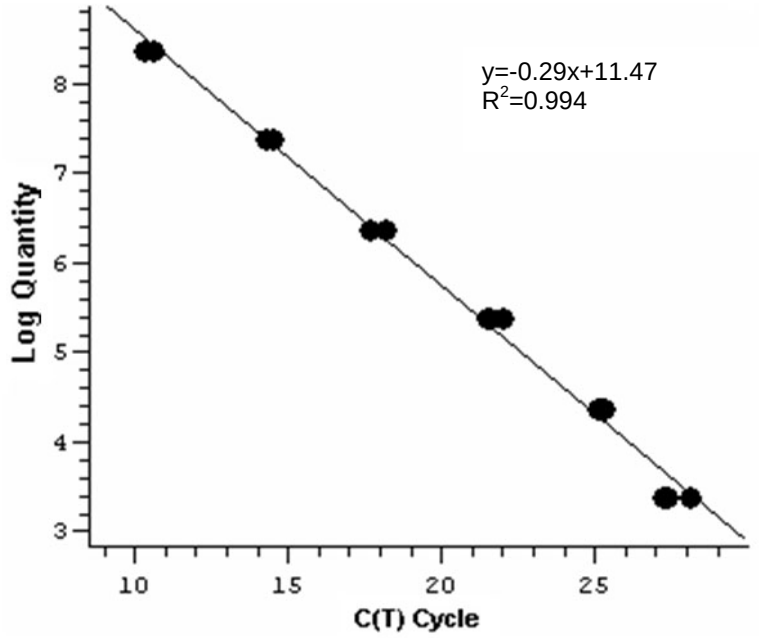

Figure D.2 **Linear regression of standard curve.**

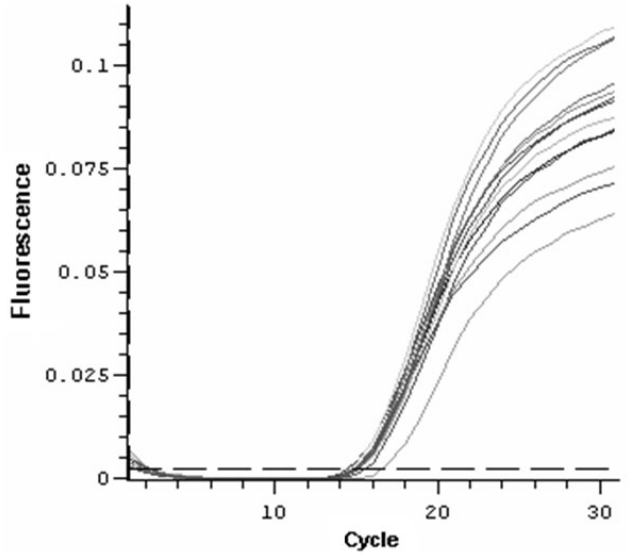

Figure D.3 **PCR sample cycle dependency of the PCR amplification expressed as the fluorescence.**

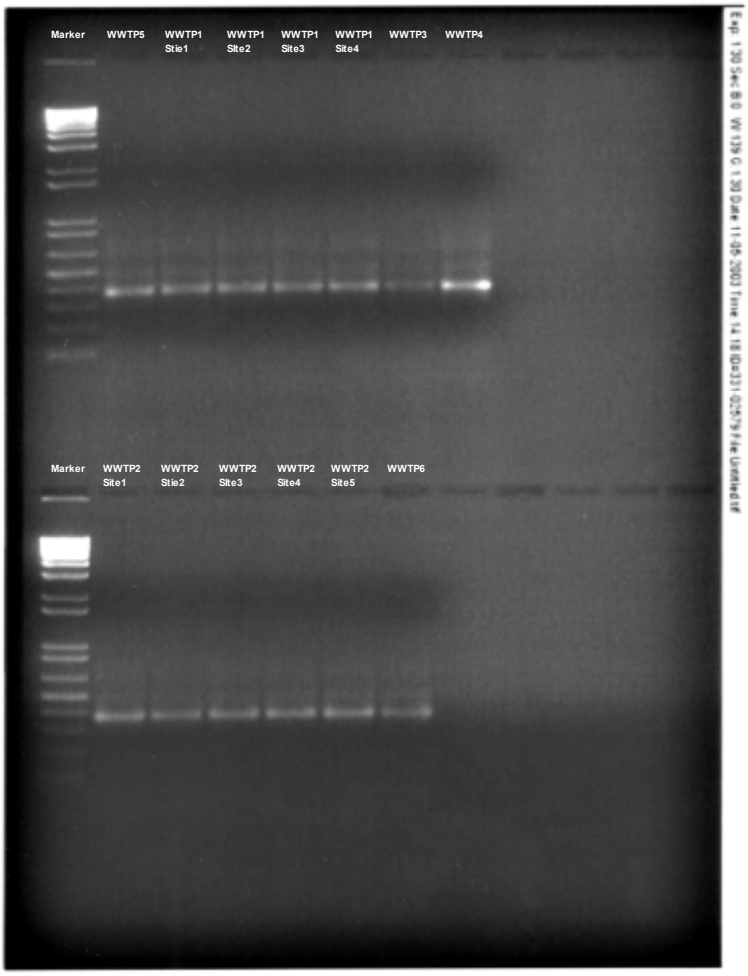

Figure D.4 **Photograph of gel purification of the activated sludge DNA samples in gel electrophoresis.**

Table D.3 **Real-time PCR experimental results**

Real-time PCR Standards					Real-time PCR Samples				
No	Type	Label	C(T)	Copies	No	Type	Label	C(T)	Copies
A1	Standard	2.29E+05	27.40689	2290	A4	Sample	WWTP5	15.53504	1.05E+07
A2	Standard	2.29E+05	27.27008	2290	B4	Sample	WWTP1,site1	15.15933	1.34E+07
A3	Standard	2.29E+05	28.12738	2290	C4	Sample	WWTP1,site2	15.0444	1.45E+07
B1	Standard	2.29E+06	25.14487	22900.01	D4	Sample	WWTP1,site3	15.00347	1.49E+07
B2	Standard	2.29E+06	25.35976	22900.01	E4	Sample	WWTP1,site4	14.51303	2.05E+07
B3	Standard	2.29E+06	25.19397	22900.01	F4	Sample	WWTP3	14.76125	1.74E+07
C1	Standard	2.29E+07	22.01048	229000.1	G4	Sample	WWTP4	15.13297	1.36E+07
C2	Standard	2.29E+07	21.55913	229000.1	H4	Sample	WWTP2,site1	15.12359	1.37E+07
C3	Standard	2.29E+07	21.57635	229000.1	A5	Sample	WWTP2,site2	15.01482	1.48E+07
D1	Standard	2.29E+08	18.18824	2290001	B5	Sample	WWTP2,site3	14.94819	1.54E+07
D2	Standard	2.29E+08	18.19804	2290001	C5	Sample	WWTP2,site4	15.05388	1.44E+07
D3	Standard	2.29E+08	17.70977	2290001	D5	Sample	WWTP2,site5	14.41029	2.20E+07
E1	Standard	2.29E+09	14.55381	2.29E+07	E5	Sample	WWTP6	16.5367	5408948
E2	Standard	2.29E+09	14.3315	2.29E+07					
E3	Standard	2.29E+09	14.28871	2.29E+07					
F1	Standard	2.29E+10	10.63472	2.29E+08					
F2	Standard	2.29E+10	10.35973	2.29E+08					
F3	Standard	2.29E+10	10.63369	2.29E+08					
G1	Blank	2.29E+04	None	N/A					
G2	Blank	2.29E+04	None	N/A					
G3	Blank	2.29E+04	None	N/A					

Table D.4 **List of organisms with 103bp in the real-time-t-RFLP analysis [1]**

Sequence ID	Organism Name
AF067729	Taylorella asinigenitalis UCD 1
Emi.hux_C	Emiliania hucleyi str. Plymouth Marine Laborator PML 92D (flagellate alga; haptophyte) – chloroplast
Ochs.sp_C	Ochrosphaera sp. Str. 181 (haptophyte) - chloroplast
X85248	Gemmata obscuriglobus ACM 2246
Ochs.nea_C	Ochrosphaera neapolitana CCMP 593 (haptophyte) - chloroplast
str.10_40	str. PUS10.40
Z98591	Leptospira biflexa subsp. Tororo str. mutant 6/1994
Isoc.sp_C	Isochrysis sp. (flagellate alga) - chloroplast
env.OM21	clone OM21
Stc.gallyt	Streptococcus gallolyticus ACM 3969 (T)
Gmt.obscur	Gemmata obscuriglobus str. UQM 2246 ACM 2224 (T)
U87816	Corynebacterium jeikeium ATCC 43216
Gmt.obscu4	Gemmata obscuriglobus DSM 5831 (T)
Y10867	Streptococcus caprinus ACM 3970

[1] The sequence ID and names were obtained from online analysis page of Center for Microbial Ecology, Michigan State University.
Website:
http://rdp.cme.msu.edu/html/TAP-trflp.html#program

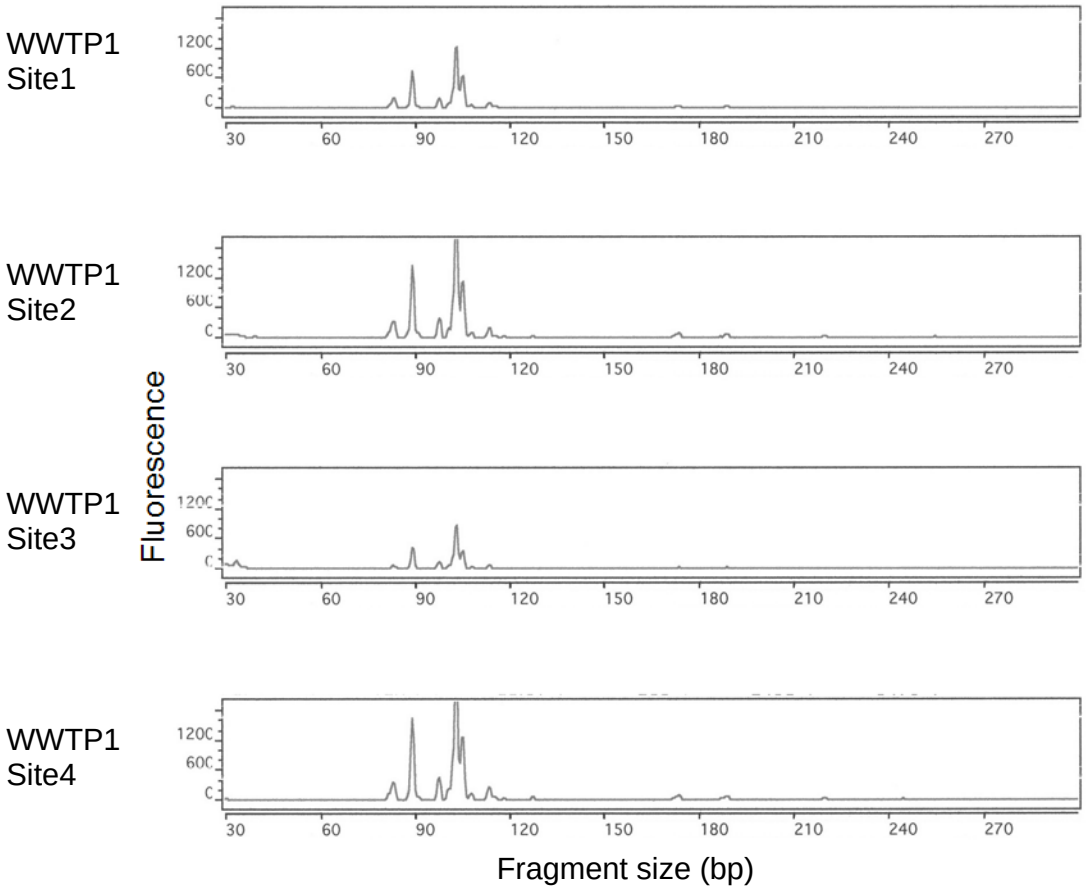

***Figure D.5* t-RFLP experimental results of activated sludge in WWTP1.**

Table D.5 **t-RFLP experimental data for the WWTP1, Figure D.5**

Dye/Sample Peak	Minutes	Size	Peak Height	Peak Area	Data Point
1G,1	12.74	83.61	203	2498	3473
1G,2	12.93	89.38	763	6378	3526
1G,3	13.21	97.96	202	1719	3603
1G,4	13.32	101.04	116	1015	3631
1G,5	13.39	103.25	1245	12826	3652
1G,6	13.47	105.36	679	7846	3672
1G,7	13.75	113.70	128	1437	3750
2G,13	12.66	83.36	337	5147	3453
2G,14	12.87	89.40	1483	12539	3508
2G,15	12.92	91.06	110	1015	3523
2G,16	13.15	97.94	422	3686	3584
2G,17	13.24	100.95	204	1822	3611
2G,18	13.32	103.28	2547	25954	3633
2G,19	13.40	105.40	1156	13483	3653
2G,20	13.49	108.08	128	1342	3678
2G,21	13.68	113.81	223	2497	3731
2G,22	15.54	173.11	84	491	4236
2G,23	15.56	173.92	103	1057	4243
2G,24	16.04	188.93	78	1027	4373
3G,24	12.83	89.46	446	3590	3498
3G,25	13.11	97.95	153	1393	3574
3G,26	13.21	101.04	93	812	3602
3G,27	13.29	103.25	902	9078	3623
3G,28	13.36	105.25	360	4296	3642
3G,29	13.65	113.71	86	1038	3721
4G,7	12.62	83.37	361	5614	3440
4G,8	12.82	89.41	1674	13995	3495
4G,9	12.87	90.96	94	782	3509
4G,10	13.09	97.83	477	4101	3570
4G,11	13.20	100.95	252	2296	3598
4G,12	13.28	103.27	2861	28999	3620
4G,13	13.35	105.29	1287	15171	3639
4G,14	13.44	107.97	149	1413	3364
4G,15	13.63	113.70	273	2772	3717
4G,16	13.67	114.79	76	681	3727
4G,17	15.51	173.79	103	1306	4228
4G,18	15.99	188.96	96	1227	4359

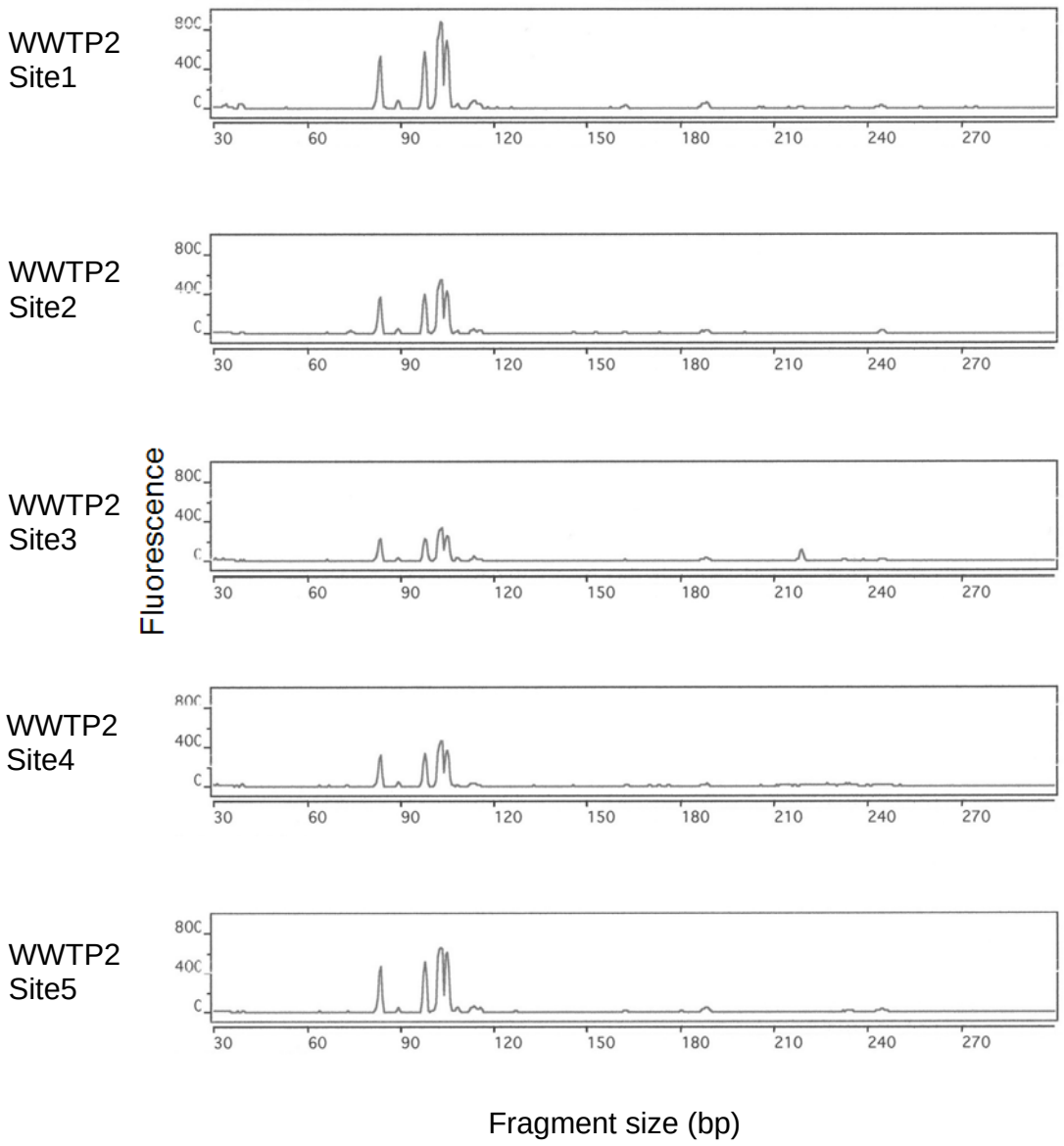

***Figure D.6* t-RFLP experimental results of activated sludge in WWTP2.**

Table D.6 **t-RFLP experimental data for the WWTP2, Figure D6**

Dye/Sample Peak	Minutes	Size	Peak Height	Peak Area	Data Point
8G,4	12.61	83.59	547	5710	3437
8G,5	12.80	89.30	93	725	3489
8G,6	13.08	97.83	586	5295	3565
8G,7	13.23	102.33	779	6148	3606
8G,8	13.26	103.18	900	7687	3614
8G,9	13.31	104.98	704	8546	3630
8G,10	13.61	113.74	92	1095	3712
9G,1	12.62	83.59	372	3642	3440
9G,2	13.09	97.83	419	3703	3568
9G,3	13.24	102.34	512	4285	3609
9G,4	13.27	103.19	572	4425	3617
9G,5	13.32	104.90	446	5205	3663
10G,11	12.62	83.52	237	2465	3441
10G,12	13.10	98.05	240	2480	3571
10G,13	13.24	102.42	318	2560	3611
10G,14	13.27	103.27	350	2720	36198
10G,15	13.33	104.96	275	3382	3635
11G,1	12.66	83.58	325	3200	3452
11G,2	13.13	97.94	343	3080	3581
11G,3	13.28	102.32	437	3384	3621
11G,4	13.31	103.17	480	3860	3629
11G,5	13.37	104.87	380	4648	3645
12G,1	12.63	83.58	482	4248	3444
12G,2	13.10	97.83	533	4502	3572
12G,3	13.25	102.32	661	5150	3613
12G,4	13.28	103.17	670	5199	3621
12G,5	13.34	104.87	626	6984	3637
12G,6	13.64	113.70	76	692	3719

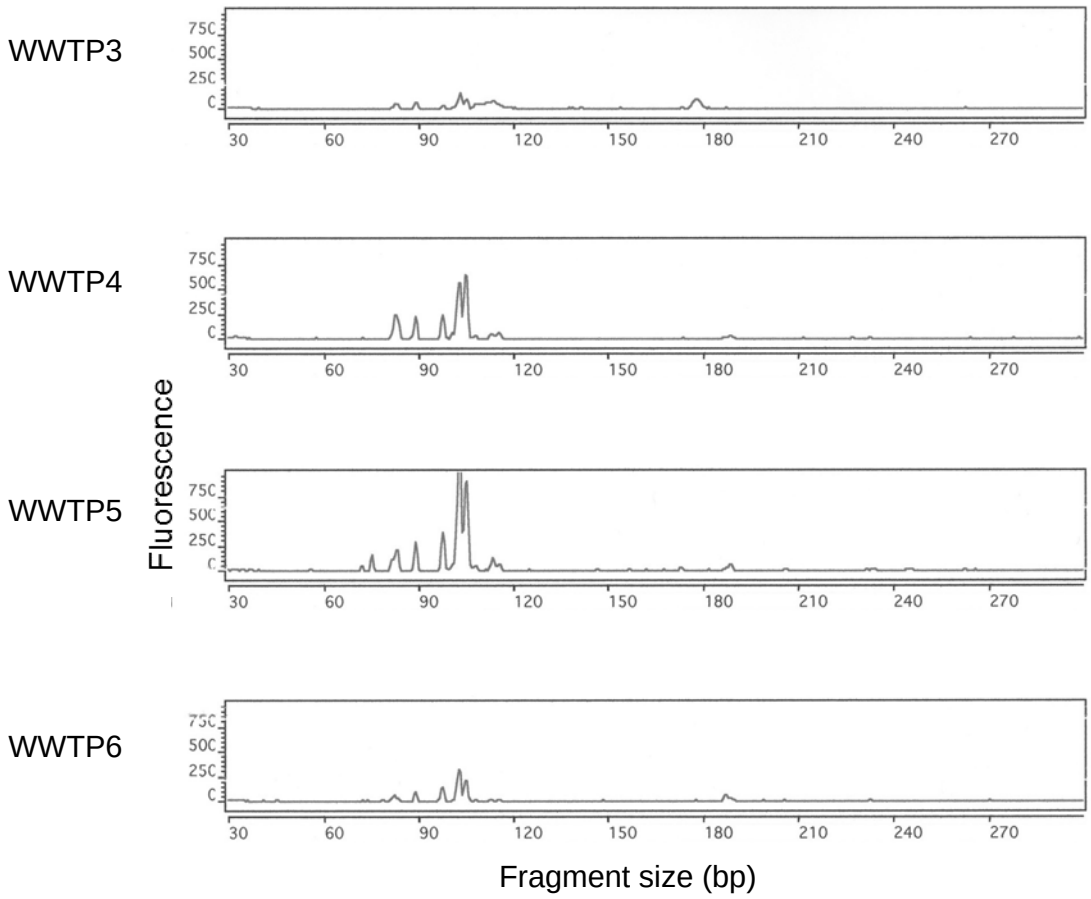

***Figure D.7* t-RFLP experimental results of activated sludge in WWTP3, WWTP4, WWTP5, and WWTP6.**

Table D.7 **t-RFLP experimental data for the WWTP3, WWTP4, WWTP5, and WWTP6, Figure D.7**

Dye/Sample Peak	Minutes	Size	Peak Height	Peak Area	Data Point
5G,1	13.61	103.49	173	2062	3721
5G,2	13.68	105.36	103	1293	3730
5G,3	13.90	111.74	80	714	3791
5G,4	13.94	112.69	75	576	3800
5G,5	13.97	113.75	89	1415	3810
5G,6	16.03	178.19	109	2585	4371
6G,1	12.59	82.94	250	4041	3433
6G,2	12.80	89.30	238	1914	3491
6G,3	13.08	97.83	253	2157	3567
6G,4	13.26	103.17	577	7476	3616
6G,5	13.33	105.19	654	7712	3635
7G,2	12.54	81.99	126	914	3420
7G,3	12.59	83.30	225	2759	3432
7G,4	12.79	89.36	295	2111	3487
7G,5	13.07	97.93	400	3271	3563
7G,6	13.24	103.17	1282	14184	3611
7G,7	13.32	105.30	912	11073	3631
7G,8	13.60	113.71	133	1512	3709
13G,3	12.81	89.29	106	679	3492
13G,4	13.09	97.83	153	1332	3568
13G,5	13.26	103.06	333	3797	3616
13G,6	13.34	105.10	221	2595	3636
13G,7	15.92	187.19	80	699	4341

Table D.8 **Calculation of t-RFLP results**

Fragment/ Area	WWTP5	WWTP1 S1	WWTP1 S2	WWTP1 S3	WWTP1 S4	WWTP3	WWTP4
76	-	-	-	-	-	-	1004
82	-	-	-	-	-	-	914
83	-	2498	5174		5614	4041	2759
89	-	6378	12539	3590	13995	1914	2111
91	-	-	1015	-	782	-	-
98	-	1719	3686	1393	4101	2157	3271
101	-	1015	1822	812	2296	-	-
102	-	-	-	-	-	-	-
103	2062	12826	25954	9078	28999	7476	14184
105	1293	7846	13483	4296	15171	7712	11073
108	-	-	1342	-	1413	-	-
112	576	-	-	-	-	-	-
113	1415	1437	2497	1038	2772	-	1521
114	-	-	-	-	681	-	-
156	-	-	-	-	-	-	-
173	-	-	-	-	1306	-	-
178	2585	-	1548	-	-	-	-
187	-	-	-	-	-	-	-
189	-	-	1027	-	1227	-	-
218	-	-	-	-	-	-	-
310	-	-	-	-	-	-	1320
313		1866	3174		4269	-	2674
327	-	-	-	-	-	7294	11602
328	-	4938	6350	2195	-	-	-
329	-	2521	6035	1435	14727	-	
337	-	-	-	-	-	-	2318
339	-	1220	2907	-	2148	-	-
351	-	-	-	-	-	-	-
352	-	-	-	-	-	-	-
Sum	7931	44264	88553	23837	99501	30594	54751

***Table D.8 C*ontinued**

Fragment/ Area	WWTP2 S1	WWTP2 S2	WWTP2 S3	WWTP2 S4	WWTP2 S5	WWTP6
76	-	-	-	-	-	-
82	-	-	-	-	-	-
83	5710	3642	2465	3200	4248	-
89	725	-	-	-	-	679
91	-	-	-	-	-	-
98	5295	3703	2480	3080	4502	1332
101	-	-	-	-	-	-
102	6148	4285	2560	3384	5150	-
103	7687	4425	2720	3860	5199	3797
105	8546	5205	3382	4648	6984	2595
108	-	-	-	-	-	-
112	-	-	-	-	-	-
113	1095	-	-	-	692	-
114	-	-	-	-	-	-
156	-	-	-	-	-	-
173	-	-	-	-	-	-
178	-	-	-	-	-	-
187	-	-	-	-	-	-
189	-	-	-	-	-	-
218	-	-	-	-	-	-
310	-	-	-	-	-	-
313	-	-	-	-	-	-
327	-	-	-	-	-	1217
328	13174	8507	6627	6210	6660	1572
329	-	-	-	-	5736	-
337	-	-	-	-	-	-
339	-	-	-	-	-	-
351	-	-	-	-	-	-
352	-	-	-	-	-	-
Sum	48380	29767	21468	24382	39171	11891

Table D.8 **Continued**

Fragment %	WWTP5	WWTP1 S1	WWTP1 S2	WWTP1 S3	WWTP1 S4	WWTP3	WWTP4
76	-	-	-	-	-	-	0.018
82	-	-	-	-	-	-	0.016
83	-	0.056	0.057	-	0.054	0.130	0.048
89	-	0.144	0.138	0.151	0.135	0.062	0.037
91	-	-	0.011	-	0.008	-	-
98	-	0.039	0.040	0.058	0.039	0.069	0.057
101	-	0.023	0.020	0.034	0.022	-	-
102	-	-	-	-	-	-	-
103	0.260	0.290	0.285	0.381	0.279	0.240	0.248
105	0.163	0.177	0.148	0.180	0.146	0.248	0.193
108	-	-	0.015	-	0.014	-	-
112	0.073	-	-	-	-	-	-
113	0.178	0.032	0.027	0.044	0.027	-	0.027
114	-	-	-	-	0.007	-	-
156	-	-	-	-	-	-	-
173	-	-	-	-	0.013	-	-
178	0.326	-	0.017	-	-	-	-
187	-	-	-	-	-	-	-
189	-	-	0.011	-	0.012	-	-
218	-	-	-	-	-	-	-
310	-	-	-	-	-	-	0.023
313	-	0.042	0.035	-	0.041	-	0.047
327	-	-	-	-	-	0.234	0.203
328	-	0.112	0.070	0.092	-	-	-
329	-	0.057	0.066	0.060	0.142	-	-
337	-	-	-	-	-	-	0.040
339	-	0.028	0.032	-	0.021	-	-
351	-	-	-	-	-	-	-
352	-	-	-	-	-	-	-

Table D.8 **Continued**

Fragment %	WWTP2 S1	WWTP2 S2	WWTP2 S3	WWTP2 S4	WWTP2 S5	WWTP6
76	-	-	-	-	-	-
82	-	-	-	-	-	-
83	0.113	0.122	0.109	0.124	0.102	-
89	0.014	-	-	-	-	0.057
91	-	-	-	-	-	-
98	0.104	0.124	0.110	0.119	0.108	0.112
101	-	-	-	-	-	-
102	0.121	0.144	0.114	0.131	0.124	-
103	0.152	0.149	0.121	0.149	0.125	0.319
105	0.169	0.175	0.150	0.180	0.168	0.218
108	-	-	-	-	-	-
112	-	-	-	-	-	-
113	0.022	-	-	-	0.017	-
114	-	-	-	-	-	-
156	-	-	-	-	-	-
173	-	-	-	-	-	-
178	-	-	-	-	-	-
187	-	-	-	-	-	0.059
189	-	-	-	-	-	-
218	-	-	0.055	-	-	-
310	-	-	-	-	-	-
313	-	-	-	-	-	-
327	-	-	-	-	-	0.102
328	0.260	0.286	0.294	0.240	0.160	0.132
329	-	-	-	-	0.138	-
337	-	-	-	-	-	-
339	-	-	-	-	-	-
351	-	-	-	-	-	-
352	-	-	-	-	-	-

Table D.8 **Continued**

Copies	WWTP5	WWTP1 S1	WWTP1 S2	WWTP1 S3	WWTP1 S4	WWTP3	WWTP4
76	-	-	-	-	-	-	3.26E+08
82	-	-	-	-	-	-	2.96E+08
83	-	1.32E+09	1.46E+09	-	2.15E+09	3.22E+09	8.95E+08
89	-	3.38E+09	3.55E+09	4.23E+09	5.37E+09	1.52E+09	6.84E+08
91	-	-	2.87E+08	-	3.00E+08	-	-
98	-	9.12E+08	1.04E+09	1.64E+09	1.57E+09	1.72E+09	1.06E+09
101	-	5.38E+08	5.15E+08	9.57E+08	8.81E+08	-	-
102	-	-	-	-	-	-	-
103	5.36E+09	6.80E+09	7.34E+09	1.07E+10	1.11E+10	5.95E+09	4.60E+09
105	3.36E+09	4.16E+09	3.81E+09	5.06E+09	5.82E+09	6.14E+09	3.59E+09
108	-	-	3.80E+08	-	5.42E+08	-	-
112	1.50E+09	-	-	-	-	-	-
113	3.68E+09	7.62E+08	7.06E+08	1.22E+09	1.06E+09	-	4.93E+08
114	-	-	-	-	2.61E+08	-	-
156	-	-	-	-	-	-	-
173	-	-	-	-	5.01E+08	-	-
178	6.72E+09	-	4.38E+08	-	-	-	-
187	-	-	-	-	-	-	-
189	-	-	2.91E+08	-	4.71E+08	-	-
218	-	-	-	-	-	-	-
310	-	-	-	-	-	-	4.28E+08
313	-	9.90E+08	8.98E+08	-	1.64E+09	-	8.67E+08
327	-	-	-	-	-	5.81E+09	3.76E+09
328	-	2.62E+09	1.80E+09	2.59E+09	-	-	-
329	-	1.34E+09	1.71E+09	1.69E+09	5.65E+09	-	-
337	-	-	-	-	-	-	7.52E+08
339	-	6.47E+08	8.22E+08	-	8.24E+08	-	-
351	-	-	-	-	-	-	-
352	-	-	-	-	-	-	-
Real-time	1.05E+07	1.34E+07	1.45E+07	1.49E+07	2.05E+07	1.74E+07	1.36E+07
DNA (ng/L)	197	175	178	189	194	142	136
copies /ml	2.06E+10	2.35E+10	2.58E+10	2.81E+10	3.99E+10	2.48E+10	1.86E+10

Table D.8 **Continued**

Copies	WWTP2 S1	WWTP2 S2	WWTP2 S3	WWTP2 S4	WWTP2 S5	WWTP6
76	-	-	-	-	-	-
82	-	-	-	-	-	-
83	1.95E+09	2.27E+09	1.94E+09	2.32E+09	3.67E+09	-
89	2.47E+08	-	-	-	-	5.56E+08
91	-	-	-	-	-	-
98	1.81E+09	2.31E+09	1.95E+09	2.23E+09	3.89E+09	1.09E+09
101	-	-	-	-	-	-
102	2.10E+09	2.68E+09	2.01E+09	2.45E+09	4.45E+09	-
103	2.62E+09	2.76E+09	2.14E+09	2.79E+09	4.49E+09	3.11E+09
105	2.92E+09	3.25E+09	2.66E+09	3.36E+09	6.03E+09	2.12E+09
108	-	-	-	-	-	-
112	-	-	-	-	-	-
113	3.74E+08	-	-	-	5.97E+08	-
114	-	-	-	-	-	-
156	-	-	-	-	-	-
173	-	-	-	-	-	-
178	-	-	-	-	-	-
187	-	-	-	-	-	5.72E+08
189	-	-	-	-	-	-
218	-	-	9.70E+08	-	-	-
310	-	-	-	-	-	-
313	-	-	-	-	-	-
327	-	-	-	-	-	9.96E+08
328	4.50E+09	5.31E+09	5.21E+09	4.49E+09	5.75E+09	1.29E+09
329	-	-	-	-	4.95E+09	-
337	-	-	-	-	-	-
339	-	-	-	-	-	-
351	-	-	-	-	-	-
352	-	-	-	-	-	-
Real-time	1.37E+07	1.48E+07	1.54E+07	1.44E+07	2.20E+07	5.41E+06
DNA (ng/L)	126	126	115	130	163	180
copies /ml	1.73E+10	1.86E+10	1.77E+10	1.87E+10	3.58E+10	9.74E+09

VITA

Yuechuan Yang was born on Dec 2, 1978 in Chengdu, China. He attended No. 7 High School and graduated in June 1997. He then pursued a Bachelor of Science degree in Civil Engineering at Hunan University. After completing his degree at Hunan University in July 2001, he joined a working group in Chengdu Municipal Engineering Company and worked there as an assistant engineer. By July 2002, he attended University of Tennessee and sought a Master of Science degree in Environmental Engineering.